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## Preface

It is our great pleasure and honour to welcome you to the 60<sup>th</sup> International Meat Industry Conference (MEATCON2019) which will be held on Mt. Kopaonik, Serbia on September 22-25, 2019, hosted traditionally by the Institute of Meat Hygiene and Technology, Serbia.

The Conference aims to serve as a mutual platform to discuss the latest trends and address critical issues in different areas of food science (Food Safety, Food Quality, Food Technology, Food related Legislations, Food & Environment, Nutrition, Sustainable production, Consumer studies) across Europe, bringing together professionals from all sectors including government, industry, research institutions and academia. This three-days gathering will cover the whole picture of animal food chain, including production and technology aspects, quality issues, consumer behaviour, safety risk and intervention strategies – with 114 contributions that have been accepted. All papers were subjected to rigorous peer-review by conference committee members, international reviewers as well as English language editorial service. It is an honour for us to publish all the contributions to the Conference in this volume and we hope that they will be very useful for the Conference participants and other potential interested readers.

In addition, the 60<sup>th</sup> International Meat Industry Conference offers an exclusive opportunity for researchers across the globe to meet, network and perceive new scientific innovations. World-renowned speakers from Serbia and abroad, oral and poster presentations, round table, innovative techniques, developments and the newest updates in food science are principal features of this Conference.

The Conference has been supported by: Ministry of Agriculture, Forestry and Water Management of the Republic of Serbia – Veterinary Directorate, Ministry of Education, Science and Technological Development of the Republic of Serbia and Chamber of Commerce and Industry of Serbia and co-organized jointly by the Faculty of Veterinary Medicine (University of Belgrade), Institute of Food Technology (University of Novi Sad) and Scientific Veterinary Institute “Novi Sad“. The Conference has been sponsored by national meat industry and other companies from the region.

Target audience:

- Food Science Technologists
- Food Science Scientists and Researchers
- Food Science and Safety Department
- Food Science Faculty
- Food Professionals from Manufacturing, Retail and Food Service Industry

On behalf of the committee, we would like to thank all authors who have contributed to this proceedings, reviewers, speakers, attendees, organizing committee and sponsors who contributed to the success of MEATCON2019.



Dr Brankica Lakicevic  
Chairman of the Programme Committee



# Why it is so difficult to end surgical castration of boars in Europe: Pros and cons of alternatives to piglet castration

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**Abstract.** Entire male pigs can accumulate sex specific off odours, i.e. boar taint, caused by the testicular steroid, androstenone, and the product of microbial protein degradation, skatole, which is formed in the large intestine. To avoid the off odour of pork and to reduce male specific behaviour, farmers have traditionally castrated their male piglets surgically and the procedure is still common in most countries. As this has usually been done without any pain relief, this measure faces strong public criticism. European stakeholders committed themselves to end this practice from 2018, but today, 75% of male piglets are still surgically castrated in the EU. Pain relief during and/or after surgery is increasingly used in some Western European countries to avoid suffering of the animals, but the surgery and risk of infections remain. Alternatives without surgical castration in pork production are raising entire males or immunocastration. Entire males have the advantage of a high growth potential and a good feed conversion rate, but the risk of boar taint and welfare problems due to male behavior limit the acceptance by the pork chain. Immunocastration reduces these problems but also decreases, in part, the anabolic advantage of males. To find country-specific, tailored solutions, there is a need to bundle the research activities along the pork chain and to spread scientific information to increase the acceptance of alternatives by farmers, industry and consumers.

## 1. Introduction

For a very long time, farmers have been castrating their male piglets surgically without any pain relief. The practice is still common in most countries, but it is increasingly facing strong criticism because of the pain associated with the surgery. The suffering incurred by the animal during the surgical process and the following days has been well documented during the last 15 years. In 2010, European stakeholders had already committed themselves to end this practice and to develop pork production systems by 2018 that are independent of surgical castration. Even though a lot of time and effort has been expended to reach this aim, and alternatives are available, 75% of male piglets are still surgically castrated in the EU (1,2). The COST action IPEMA (Innovative Approaches for Pork Production with Entire Males) aims to establish alternatives to surgical castration in pork production in Europe and bundles the research activities along the pork chain to find tailored solutions for the different countries.

## 2. Why are piglets castrated?

The traditional reason for castration of boars is the presence of boar taint, an offensive odor and flavor observed in the meat from some entire male pigs. Two main compounds are held as responsible for boar taint, androstenone and skatole (3). Both compounds are lipophilic and thus accumulate in



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adipose tissue of growing boars around the most common slaughter stages due to the progressing pubertal development and can lead to consumers' complaints (4).

Androstenone, is a testicular steroid with a urine-like smell. It has biological significance as a male pheromone and is formed in parallel to the synthesis of anabolic testicular steroids in the Leydig cells.

Skatole is a metabolite of the amino acid tryptophan with a fecal odor and is synthesized in the colon by microbial degradation. Boar carcasses can have higher skatole levels in adipose tissue than barrows or gilts because the hepatic degradation of skatole is reduced, due to lower activities of CYP2E1 and CYP2A enzymes if concentrations of androstenone, testosterone or 17-  $\beta$ -estradiol are high (5, 6, 7, 8).

A peculiarity of one boar taint compound is that about one third of the consumers is anosmic to androstenone whereas another third of consumers is highly sensitive and rejects pork with already low androstenone concentrations (9). Such a high variability in perception does not exist for the other compound, so skatole-tainted carcasses are refused by most consumers if the levels are high (10). Early castration avoids boar taint accumulation and also prevents undesirable male aggressive and sexual behavior during the fattening period.

### 3. What are the alternatives?

There are currently three possible alternatives with practical relevance: surgical castration with anesthesia and or analgesia, raising entire males, and immunocastration. Castration with chemicals injected in the testes is too painful to be considered and sperm sorting for producing only males is not practically feasible at a large scale in the pig species (11, 12).

During recent years in Western Europe, some kind of **pain relief during and/or after surgery** is increasingly used due to societal and market pressure.

Since the 1960s, farmers have been **raising entire males** in the UK and Ireland. Castration was also abandoned in Spain and Portugal for mainstream standard pig production, while it is still performed in the high quality production systems. Entire male pigs now constitute a sizeable part of pig production in the Netherlands, Belgium, Germany, and France.

**Immunocastration** has been developed to a significant degree only in Belgium. In most Eastern European countries, piglet castration is not an issue yet, although immunocastration is under consideration to reduce feed costs and fat content. (13, 14)

#### 3.1 Surgical castration with pain relief during and/or after surgery

General anesthesia is effective in preventing pain during castration but not in relieving post-operative pain. Conversely, analgesia is effective post-surgery but not during it. Only combined anesthesia and analgesia is fully effective for pain avoidance, but it is a costly procedure, especially if vets are required. General anesthesia for piglet castration is administered via inhalation ( $\text{CO}_2$ , isoflurane) or intramuscularly (Ketamine).  $\text{CO}_2$  is cheap but aversive to the animal (11, 12). Isoflurane is efficient but costly and it can affect workers and the environment. Ketamine is risky for the animal and requires a lot of monitoring. Local anesthesia with Lidocaine injected in the testes is effective if carefully performed to avoid pain during the injection. Procaine is less efficient than Lidocaine, but in several countries Procaine is the only approved medication for local anesthesia in pigs. The main drugs used for analgesia include Meloxicam, Flunixin and Metamizole (13).

The advantages, however, are that quality problems due to boar taint or changes fatty acid composition are prevented, and the carcasses are suitable for all traditional pork products (13, 15). Additionally, management is easier and welfare higher than in entire males, as sex specific sexual and aggressive behaviors are abolished. The disadvantages are that surgery is still required and the risk of post-surgical wound infections persists (16). Also the anabolic potential of barrows is about 10-20% lower than that of boars, and the barrows' feed consumption is high. Thus, a total of 10 to 15% more feed is required to produce the same amount of meat from barrows than from boars, and nitrogen excretion is about 15% higher than in entire males (13, 18).

### 3.2 Entire male pigs

Leaving the male pigs entire avoids a cumbersome job, and is highly efficient later on, as anabolic testicular steroids increase feed efficiency and muscle content of the carcass as well (13, 18). However, it also has drawbacks. Some farmers have difficulties in managing the more restless entire males, which exhibit mounting and aggressive behavior. The long-lasting reduced welfare of the animals harassed by their dominant pen mates is to be compared with the short duration acute pain experienced by all animals during castration. Penile injuries are also quite common (14, 19, 20, 21). The increased activity of entire males in the pre-slaughter period results in more frequent carcass lesions and dark-firm-dry (DFD) meat. Lower fat content and increased fat unsaturation are detrimental for processing dry-cured products (13, 15). Finally, the occurrence of boar taint is a serious potential risk for consumer satisfaction (10,22, 23). Processing – except dilution with untainted meat – has only limited effects on increasing the quality of the final product, as further explained in specific contributions at this meeting.

Boar taint of carcasses can be reduced to some extent using a combination of genetic, dietary and management methods. For example, skatole can be efficiently controlled by feeding measures, such as the addition of inulin to the feed, but these measures are not yet efficient in controlling androstenone levels and, thus, do not guarantee boar taint-free populations (24, 25). Genetic selection for low boar taint levels has already been included in some breeding programs. Although the heritability of both androstenone and skatole levels allows efficient selection of low boar taint lines, the interdependence with the regulation of fertility traits and growth has to be considered. New techniques and strategies are promising but do not provide a rapid solution (26, 27, 28). Also transport and treatment before lairage have significant effects in boar taint compounds in the carcass of entire males, so the effort of all preceding management steps can be spoiled by inappropriate handling and transport (29). On-line assessment of boar taint is possible, with additional costs (30). The “human nose” method is simple and cheap but its effectiveness in protecting consumers from dissatisfaction is not documented in scientific publications (31). Instrumental methods are close to the market (30).

### 3.3 Immunocastration

Two shots of anti GnRH vaccination in at least a 4-week interval are required to effectively postpone sexual development and decrease boar taint. Male pigs are vaccinated at least twice (at an age of 8-12 weeks and 4 to 6 weeks before slaughter) during the fattening period to suppress the hypothalamic pituitary gonadal axis (32, 33). The vaccination results in production of antibodies against the hypothalamic hormone GnRH, which is a key hormone in the endocrine cascade regulating testicular functions. From a few days after the second vaccination, testosterone secretion ceases and the animals behave like castrates and increase their feed intake (33,34). The advantages are improved welfare by circumventing many of the concerns with physical castration methods. A major benefit of immunocastration is preventing the pain associated with the castration procedures and the risk of wound infection. Additionally, the behavioral effects and a reliable reduction of boar taint compounds favor this measure (19). The disadvantages are that additional costs for the vaccine have to be considered and feeding costs and carcass quality are only intermediate between those observed in entire males and castrates (13, 18, 35). The longer the delay between the second vaccination and slaughter, the closer the performance is to that of castrates (36). Immunocastration is common in Oceania and South America, but its development in Europe is still impaired by a strong reluctance from chain actors, based on assumed rejection of the practice by the consumers (13, 14). The main argument is an irrational fear that consumption of pork from immunocastrates could affect human fertility. The safety for consumers, however, is well documented (37, 38). The antigenic GnRH fragment of the vaccine has only a potency of 0.2% on LH-release when compared to injections of the decapeptide. The carrier protein is used also for other vaccines and has no toxic neither hormonal activity. The construct of the GnRH-fragment conjugated to the carrier protein has no hormonal activity at all, not if it is administered orally and nor if it is injected. Thus, the only fact-based risk is the accidental self-injection of the person applying the vaccine. As a second vaccination is crucial for

successful immunocastration, the on-farm risk seems to be manageable. Irrespective, fact-based communication about the pros and cons of immunocastration are required to prevent adverse farmer and consumer reactions.

#### 4. Conclusions

The castration issue is a good example of conflicting aims in pork production and needs scientific progress and good communication along the pork chain to find country-specific, tailored solutions. Whereas in the declaration of Brussels, stakeholders of the pork chain committed themselves to end surgical castration, today, 75% of male piglets are still surgically castrated. The main reasons are that quality and welfare problems of entire male pigs have not been solved reliably. To reduce the welfare problems, pain relief during and/or after surgery is increasingly used in some Western European countries. However, the surgery and risk of infections remain. Alternatives without surgical castration in pork production are raising entire males or immunocastration. Entire males have the advantage of a high growth potential and a good feed conversion rate, but the risk of welfare problems, in addition to quality problems due to boar taint and adipose tissue composition, limit the acceptance by the pork chain. Immunocastration reduces these problems but also decreases, in part, the anabolic advantage of males. To find country-specific, tailored solutions, there is a need to bundle the research activities along the pork chain and to spread scientific information to increase the acceptance of alternatives by farmers, industry and consumers.

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# Attitudes and beliefs of consumers towards pig welfare and pork quality

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**Abstract.** The aim of this study is to provide an overview of consumer attitudes and beliefs regarding pig welfare and quality of pork meat. Regarding animal welfare, several aspects of farm management and practice were considered, with an emphasis on alternatives to surgical castration without pain control, such as entire male production, castration with anaesthesia and immunocastration. Organic production was also considered. For meat quality, aspects of sensory quality were studied, specifically those related to boar taint, again including organically produced meat. A wide variety of consumer attitudes and beliefs are revealed in these studies as being strongly dependent on culture and influenced by information provided. These insights can be used when producing and commercializing pork and pork products as well as when developing marketing strategies to reach various consumers and satisfy market demands.

## 1. Introduction

Consumers are the last step in the consumption chain, and thus, it is important to understand their behaviour, which is affected by psychological, sensory and marketing factors [1]. When studying consumers' behaviour it is important to consider that the relationship between citizen attitudes and consumer attitudes is weak [2]. Citizen attitudes are mainly determined by information, process characteristics, beliefs and feelings. When citizens assume the role of consumer, their attitudes and beliefs can diverge. Consumer attitudes and beliefs are mainly determined by price, taste, health and convenience. Beliefs are the cognitive knowledge related to information that an individual has about something that can be linked to some attribute, action or event. Beliefs can be obtained by either direct observation or experience, provided from outside or inferred. Feelings are affective responses of an individual and are responses towards an object, a person, an issue or an event [3].

This paper focuses on factors related to the type of pig production system, including animal welfare, the specific case of alternatives for piglet castration and organic farming and their impact on sensory and meat quality and willingness to pay.

Several definitions can be found for animal welfare. In general, they all include the (absence of) suffering and level of satisfaction of the animals during their entire life, including at slaughter. One important welfare issue in pig production is castration of male piglets. Worldwide, the majority of the male fattening pigs are surgically castrated, usually without anaesthesia. Castration is performed to reduce the levels of androstenone and skatole in the meat. These are the main compounds responsible





for boar taint, which causes an unpleasant odour and taste in heated meat of entire male pigs. Castration also makes it easier to handle the pigs, as castrates exhibit less aggressive and sexual behaviour than entire males. Pain control during castration by means of analgesia and/or anaesthesia is required in several EU countries. One alternative to castration is immunocastration, a procedure that uses vaccination against endogenous gonadotropin releasing factor to achieve castration-like physiological effects. Production of entire males is another alternative [5]. Slaughtering entire males could increase the risk of boar-tainted carcasses entering the market, as currently no objective boar taint detection system is yet available at slaughterhouses. The negative sensory experience of boar taint in the kitchen can negatively affect consumer acceptance of pork meat. The case of piglet castration and its alternatives is, therefore, interesting to study, as it includes aspects of animal welfare as well as perception of sensory quality, food safety and possibly also the price of meat products. Consumer knowledge regarding piglet castration is generally low but a higher number of consumers are aware of organic production.

The objective of this study was to obtain an overview of consumer attitudes and beliefs towards some aspects related to animal welfare and meat quality, with an emphasis on alternatives to surgical castration without anaesthesia, as well as organic pork production.

## **2. Attitudes and beliefs of consumers towards animal welfare**

Animal welfare has many suggested definitions but most of them consider the level of suffering and satisfaction of the animals. Animal welfare can be evaluated on-farm, during transport and at the slaughter plant. Citizens' concerns about have increased in the last years, but in general, consumers report having a low level of information about animal welfare and its attributes [6], and only some of them think about animal welfare when buying pork [7]. According to the review of Thorslund *et al.* [8], concerns about animal welfare can be related both to the naturalness (i.e. space allowance and freedom) and to the level of suffering. In Europe, most of the consumers know little about the conditions under which animals are raised, although they believe the conditions for the animals in their country have improved in the last 10 years [9]. Furthermore, some consumers associate better animal welfare with better meat quality [9][10].

### *2.1. Production aspects*

The perceived importance of animal welfare varies depending on which stakeholders of the production chain are considered. In this sense, differences in perceived importance were found between citizens and farmers regarding aspects of housing and climate, ability to engage in natural behaviour, animal health, transport and slaughter, feed and water, animal suffering and stress, and human/animal relationship. Differences were mainly related to the animals' ability to engage in natural behaviour and in aspects related to animal production where the experience of the farmers gives the citizens a different perspective [11].

In a study carried out with German consumers, "The sow can walk around freely instead of being restrained for most of its life" was the most chosen attribute of pork (41.5%) when shopping. With the same study performed in Poland, the most chosen attribute (49.4%) was "The pigs are guaranteed free of microbial contaminants like *Salmonella*". Besides the interest in sow welfare found in Germany, the other most-chosen attributes were related to food safety. In both countries, four clusters of consumers (each with a different attitude), were identified. The first cluster was defined as being interested in production (environment and farm) issues, the second in fat content and colour attributes, the third cluster focused mainly on price and the last mainly on the origin of the pork [12]. In other research conducted with consumers from Belgium, Denmark, Germany and Poland, which aimed to determine how attitudes are affected by characteristics of pig production, the factors "housing and floor type" and "efforts to protect soil, air and water on the farm" were the most important. Housing with outdoor access was the most preferred, followed by litter housing, while slatted floor housing was the least preferred. That study again identified four consumer segments. The one supported by the largest portion of consumers (59.1%) had indifferent attitudes towards animal welfare, environmental

protection and industrial food production while only 12.3% of consumers, mainly from Germany and Denmark, belonged to the segment highly aware of animal welfare [2]. In Serbia, greater awareness towards animal welfare were found in the statements “animal welfare should be strictly controlled”, “increased regulation on the treatment of farm animals is needed in Serbia”, “animal welfare should be guaranteed by specific label” and “high animal welfare standards are necessary to guarantee the quality and safety of food”. Clustering the consumers revealed that 35% of consumers are indifferent towards animal welfare, 30% of them are truly interested in animal welfare, 25% know where to find animal welfare-guaranteed products and 10% of them are antagonistic towards animal welfare [13]. In a study carried out in 13 Eastern European countries, results show notable country-based differences in attitudes and beliefs towards animal welfare, whereas a cross-country cluster analysis divided consumers in three groups, the first one (35% of consumers) “highly concerned about animal welfare”, the second one (43%) “indifferent towards animal welfare” and the third one (22%) “somewhat concerned about animal welfare” [14].

Consumer willingness to pay is an important factor when studying meat produced under higher animal welfare standards. The review of Clark *et al.* [15], using 54 studies and including data from 17 countries, mainly from Europe and the United States, studied willingness to pay for animal welfare. Although the results were not conclusive, they show that consumer willingness to pay is only half of a standard deviation higher for meat produced with attention for animal welfare. Willingness to pay depends on the country or region. This cultural diversity should be considered by policy makers and can be relevant when developing marketing strategies. Socio-demographic characteristics also affect willingness to pay, because this decreases with age, is higher for women than for men, and increases with income and education level. This diversity could also be seen in a European (EU-28) survey, where the percentage of consumers willing to pay more for animal welfare meat varied between 22 and 93%, whereas in a recent study carried out in Eastern Europe, willingness to pay varied between 3.8 and 5.2 on a 7-point scale [14].

## 2.2. *Alternatives to surgical castration: entire male production, anaesthesia and immunocastration*

Surgical castration of piglets without analgesia/anaesthesia is experiencing growing public criticism due to pain experienced by piglets during the procedure [17][4]. Non-anaesthetised surgical castration of piglets is gradually being phased out in Europe, often voluntarily, and alternatives like rearing of entire males, immunocastration and surgical castration with anaesthesia are being introduced. The consumer acceptability of these alternatives is still poorly investigated [5], and differences in study set-up and results make it difficult to draw straightforward conclusions from the few studies published. In general, consumers are not greatly aware of the boar taint issue and the methods available to avoid it, while the majority do not even associate pork with castration [6][18]. A wide study on consumers from six EU countries revealed that people do not perceive pig castration to be a relevant aspect of animal welfare, nor are they aware of its relationship with pork quality. Most consumers do expect the availability of healthy, safe and tasty meat, and therefore, factors like boar taint in entire male production could be an important issue for consumer acceptance [6]. Regarding the alternatives, European stakeholders involved in the pig production chain (i.e. veterinarians, producers, slaughterhouse operators, meat processors and others) prioritised surgical castration with anaesthesia and rated the prospects of immunocastration as low, mainly because of fear of a negative consumer response, which was identified as one of the main disadvantages for implementation of that method [5]. In general, consumer acceptance of surgical castration with anaesthesia seems to be the highest, followed by entire male production. Immunocastration rates second-lowest, with surgical castration without pain relief in last place [5]. The studies showed rather large differences in acceptance or preference across Europe. In Switzerland, surgical castration with anaesthesia was found to be the most acceptable, while immunocastration was disfavoured [19]. Norwegian consumers preferred surgical castration with anaesthesia more than immunocastration (the latter ranked higher than entire male or surgical castration without anaesthesia [20]). It is worth noting here that anaesthesia is required when performing surgical castration in those two countries. Swedish consumers evaluated

immunocastration more positively than rearing entire or surgically castrated males [21], which was similar to Belgian consumers, but only after having received more information about the alternatives [22]. Alternatives were ranked similarly (i.e. surgical castration with anaesthesia > entire male > immunocastration > surgical castration) by German consumers of organic pork [23], whilst the percentage of participants unwilling to pay more were the highest for immunocastration (48%) and surgical castration (41%) compared to entire male (21%) and surgical castration with anaesthesia (14%).

In a study carried out in 13 Eastern European countries, the results show there is probably a low level of awareness about castration of pigs, and that differences between countries can be important. For instance, in the statement ‘I think that surgical castration of pigs is easy (1) or difficult (7)’ the lowest score was for consumers from Bosnia and Herzegovina (3.1) and the highest was for Hungarian and Ukrainian consumers (5.7 in both countries). As for the attitudes and beliefs towards castration, three segments of consumers were identified: the first (47% of consumers) ‘against castration’, the second (31%) ‘indifferent towards castration’ and the third one (22%) ‘pro-castration’ [24].

While some consumers favour immunocastration, others question the practice mainly from a food safety standpoint [25] or their preference for reduced use of pharmaceuticals [26]. A guarantee of food safety and the level of food safety are important consumer concerns [20][25][27]. Norwegian consumers [20] were, in general, positive about immunocastration (for 51% it was completely acceptable, for 23% possibly acceptable, 16% did not know), while 10% found immunocastration unacceptable. The main reasons for rejection were fear of residues, fear of unknown long-term effects and unnaturalness, and their preference for the method currently in use (i.e. surgical castration with anaesthesia). The scepticism toward immunocastration did not decrease when more information on food safety was provided (i.e. there are no residues, no risk for human safety). On the other hand, the reason for the relatively high acceptance of immunocastration was the trust that Norwegian consumers have in their national food safety authority. Similarly, Italian consumers [26] accepted immunocastration even for traditional products, when institutions guarantee a strong involvement in quality, safety and ethical treatment. Swedish consumers showed no aversiveness towards immunocastration when compared to surgical castration [20], indicating the importance of animal welfare concerns over biotechnology aversion or perception of food safety risk.

### *2.3. Organic production*

Organic production is one of the production systems perceived as being related to high animal welfare requirements and expectations. Organic production is regulated by the European legislation [28], stating that in organic production “physical castration shall be allowed in order to maintain the quality of products and traditional production practices, but only by applying adequate anaesthesia and/or analgesia and by carrying out each operation at only the most appropriate age by qualified personnel”.

Consumers, especially Danes and Swedes, consider animal welfare and food safety the most important quality characteristics of organic meat. They believe that animals produced organically have access to outdoor areas and better feed and that the meat is safer because it is free of antibiotics [8].

Organic farming is perceived as being more ethical and more sustainable than conventional farming by most of the organic producers. Their motivation to produce organically is related to environmental, ethical and societal concerns. Furthermore, organic production can satisfy consumer and market demands [29].

## **3. Attitudes and beliefs of consumers towards meat quality**

Meat quality is a broad concept that includes at least the aspects of sensory quality (visual appearance, tenderness, juiciness, aroma, flavour), technological quality (water holding capacity, pH), nutritional quality (content and composition of fat, proteins, vitamins, micro- and macro-nutrients), and safety-related quality traits (microbiological and toxicological aspects). In addition, meat quality includes the socio-ethical value of the product (including production system, animal welfare and environmental aspects). Meat quality is affected by several factors, and the perception of quality can differ among

and within the different stakeholder groups. For instance, for retailers and the restaurant/catering sector, the most important quality criteria when purchasing pork meat was price, while butchers prioritise food safety, and consumers value quality the most. For consumers, quality was followed by price, origin, fat content and freshness. Butchers were the only ones that included sex category of the pig as a quality criterion [30].

As explained before, meat quality includes several points of view. Consumer acceptance and perception of pork are affected by the sensory quality of the meat, which in turn depends on numerous different factors, such as genotype, sex, *ante-mortem* and *post-mortem* treatment and processing.

### 3.1. Colour and fat content

Colour is one of the most important sensory attributes because consumers relate it to freshness [31] and shelf life [32]. In a study involving 23 countries, Ngapo *et al.* [33] showed that colour was the most important characteristic of pork compared to fat cover, marbling and drip. Nevertheless, preferences for colour varied between countries. In some countries like Australia, Ireland and Poland, light pork was clearly preferred to dark, while the opposite was observed in Taiwan.

Fat content (either fat cover or marbling) also affects consumer perception of meat. A thin external fat layer on pork was most preferred in countries like Poland, Finland, Mexico and the Netherlands while none of the 23 studied countries showed a strong consumer preference for a thick fat layer [33]. Even though marbling was not the most important parameter for the majority of the consumers, some consumers, like those from Korea, Taiwan and Japan preferred marbled pork while pork without marbling was preferred in Ireland and Australia [33]. In Spain, one segment of consumers clearly preferred marbled meat while others preferred it less marbled. It is also important to note that, although consumers can be differentiated according to their preference for marbled meat, in blind taste tests, they mainly preferred marbled pork compared to lean pork [34]. Marbling affects the tenderness of the meat, which is an important sensory attribute of pork [35]. The preference for leaner meats can be due to consumer perceptions of fatty meat as being less healthy than leaner meats [36][37] and because health is an important criterion for some consumers [38].

### 3.2. Taste and flavour: boar taint

Taste/flavour and odour are also important sensory attributes of meat. In fact, taste is the most important factor that affects consumer satisfaction for pork and pork products [39] and is the most important criterion for purchase [40]. Because the sex of the pig can have a considerable impact on the sensory quality, boar taint (an unpleasant odour/flavour) is an important issue. Surgical castration and immunocastration both reduce or eliminate boar taint [41]. Several studies have shown consumer acceptability of boar meat is affected by the boar taint levels while other studies present different conclusions. Moreover, the acceptability depends on consumer's sensitivity to androstenone [42] and skatole [43][44] and to repeated exposure to androstenone [45]. As mentioned above, consumers are generally unaware of piglet castration and the boar taint problem [27]. However, the type and the amount of information provided could affect their attitudes [22]. Some consumers report having detected boar taint when eating pork (16.5% in Switzerland) and consequently, some of them have decreased their pork consumption [19]. In addition to the possible presence of boar taint, pork from entire males is generally considered to be less tender and juicy compared to pork from castrated males, with meat from immunocastrates rated as intermediate.

Swedish consumers preferred conventional pork from surgically castrated pigs more than pork from entire boars [20], showing the importance of food quality in their decision making. As suggested by Heid and Hamm [23], differences in preference could be related to the lack of familiarity with boar taint, as most participants of their study were unfamiliar with this off-odour. Nevertheless, the participants of the focus group study of Fredriksen *et al.* [20] indicated the presence of boar taint or reduction of pork quality would induce them to reduce their pork consumption. Possibly, differences in background information provided to the consumers are the basis for these attitudes. Overall, a real sensory experience when conducting attitude surveys can clarify this difference. As reported by Kallas

*et al.* [6], those consumers that were able to differentiate meat from entire males from meat from surgically castrated pigs or considered the odour during cooking as unpleasant were willing to pay more for avoiding boar taint. Sensory experience (eating meat with boar taint or an unmasked meat product) significantly decreased the degree of randomness and consumer uncertainty [46].

### 3.3. Organically produced meat

EU legislation on organic production [42] defines organic products to be of high quality because they are produced with 'the observance of high health, environmental and animal welfare standards'. Organic production has been considered as an important quality cue for some consumers because they relate it to good taste (quality) and higher animal welfare [10]. In fact, expectations of consumers towards sensory quality of organic meat (and free-range pork) is higher than towards conventionally-raised pork, and they would also pay more for it (although not much more). However, the experienced quality (i.e. after tasting the meat) was not in agreement with consumer expectations. Consumers found the organic pork to have a slightly lower quality in terms of taste, juiciness and overall acceptability, but tenderness was rated equal [47], although tenderness could possibly decrease in organic pork due to higher physical activity [48].

Consumers also perceive organic meat as being healthier to eat than conventionally-raised meat [40]. Organic production has been associated with domestic meat [40], while domestic meat is considered to be healthier and of good quality [10]. Hemmerling *et al.* [49][47], when reviewing 58 papers about organic production, described protecting human health as the most important purchase motive and product attribute (34), followed by taste (9), environmental protection (7), no/less chemicals/pesticides (7) and safety (3). Thus, health is an important point that affects the choice of the consumers and their preferences for organic meat. Saba *et al.* [38] divided consumers in three groups according to their interest in human health: low, medium and high. The higher the interest in human health, the lower the consumption of red meat and preserved and processed meat products. Such was the case with students from New Zealand, perceiving meat as less healthy than vegetables and fruits. Furthermore, the lower the interest in health, the lower the ranking of organic food. When comparing meats from different types of animals, Italian consumers perceived pork as being less healthy to eat and having more calories than lamb, beef and chicken [50].

## 4. Conclusions

Differences in consumer characteristics and in the information received may affect attitudes and beliefs towards animal welfare and meat quality. It is difficult to compare results between studies because information provided is different among studies and this information influences consumers' opinion. Several segments of consumers can be found according to their attitudes and beliefs, thus it is important to know the characteristics of these consumers. This information can be used when producing, commercializing, and consuming meat and meat products, as well as to develop marketing strategies to reach all the consumers and to satisfy market demands.

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# Slaughtering of entire male pigs seen from the slaughterhouse perspective

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**Abstract.** Several challenges need to be taken into account before a full transition to slaughtering entire male pigs. To optimize welfare, entire male pigs should spend as short a time as possible in the pick-up facilities at the farm. At the slaughterhouse, there is no need to control the ratio between genders in the pens, as this has only minor effects on welfare. Boar taint detection must be performed, and an analytical on-line method is being developed. Rejection limits for skatole and androstenone must be set as a balance between the risk of consumers getting a negative experience on consuming the meat and the number of carcasses discarded. Smoking is a promising strategy for use of the discarded carcasses, and as much smoke as possible is recommended. Using spices and serving the meat in complex meals can also be a strategy to utilize boar tainted meat. In addition to boar taint, meat from entire male pigs is less tender and the protein content is lower than meat from castrates. In addition, the weight distribution of the carcass is different, with entire males having approximately 500g more weight at the fore-end and less at the ham. However, the meat percentages are higher in the entire males, and this can somewhat counteract the weight distribution.

## 1. Introduction

The number of entire male pigs raised and slaughtered in Europe is increasing, and the pressure from customers interested in animal welfare is further accelerating this development. However, seen from a slaughterhouse perspective, there are several challenges that need to be taken into account before a transition towards slaughtering of entire male pigs can be initiated. This review will discuss these challenges from the point when the pigs arrive at the slaughterhouse and until the meat is consumed. This includes aspects of handling the live animal to optimize animal welfare, analysis for boar taint components and how to set rejection limits, how the carcass of entire male pigs differs from castrates, and how the tainted meat can be utilized. Last, the monetary cost of slaughtering entire male pigs is calculated under different scenarios.

## 2. Animal welfare at the slaughterhouse

Entire male pigs are known to be more aggressive than castrates [1], and this must be taken into account when handling the pigs on the day of slaughter. It is recommended entire male pigs spend as short a time as possible in the pick-up facilities at the farms to reduce the frequency of skin damage before they arrive at the slaughterhouse. Keeping pigs in smaller groups during lairage at the slaughterhouse is a well-known method to reduce fighting. Despite having the pigs in smaller groups, pigs in pens with entire male pigs have more aggression instances and a lower rest index than pens





without entire male pigs. However, the observed difference in rest index between pens with and without entire male pigs is limited. Therefore, from an animal welfare point of view, it will be more important to avoid excessive handling of the animals, which would be needed if genders are kept at specified ratios in the pens. Consequently, the ratio between genders in the pens during lairage should not be controlled [2]. To increase welfare and reduce the amount of skin damage, slaughter of the unrestful pens at lairage in as fast a time as possible is recommended.

Another item incurring public attention concerning animal welfare is the need for tail docking in pigs. In production of entire male pigs, the risk of tail bites must be addressed. The incidence of tail bites in a production unit could be continuously monitored using a vision based system to assess the degree of tail bites on all carcasses at the slaughterhouse [3]. Farmers can also use this feed-back as a management tool to monitor smaller incidences of tail bites and act if the level increases.

### **3. Analysis of boar taint compounds and rejection limits**

To avoid the risk of selling boar tainted meat to the consumers, boar taint detection must be performed on all entire male carcasses. In countries like the Netherlands and Germany, this is done by using a human nose methodology in which a person smells a heated sample of fat from each entire male carcass and classifies it into acceptable/not acceptable. However, this is a rather unspecific method [4], and it is difficult to set reliable rejection limits. The quantitative, chemical analysis of the boar taint compounds skatole and androstenone is, therefore, an optimal approach to detect boar taint on the slaughter line. An analytical method based on the extraction of relevant boar taint compounds from pig backfat, followed by measurement by laser diode thermal desorption-tandem mass spectrometry is expected to be introduced at the end of 2019. This method will be able to selectively quantify the boar taint compounds, skatole and androstenone, at the pace of the slaughter line. Regardless, even if the concentrations of skatole and androstenone are known, a decision of where to set the rejection limits must be made. Several papers have been published, including consumer studies as reviewed by Font-i-Furnols [5], to help in setting sorting limits.

However, a precise acceptability level of boar taint is difficult to set and depends on several factors. A recent European study showed that consumers always preferred meat from castrates irrespective of the level of skatole and androstenone in the backfat of entire male pigs [6], and it seems unrealistic to establish a rejection limit ensuring that all consumers will like meat from entire male pigs as well as meat from castrates. This was confirmed in a Danish study estimating the risk of disliking the meat, which showed that almost 80% of the carcasses would be discarded if the risk was set at similar levels for castrates and entire male pigs [7]. Instead, a recommended approach can be to consider the acceptable risk for a negative consumer experience balanced with the percentage of discarded carcasses that would be acceptable to the slaughterhouse.

### **4. Carcass composition**

Entire male pigs have a higher meat percentage than castrates, and in most cases, this can be regarded as a positive trait. However, at the same time, the weight distribution of the three-parts (fore-end, middle and ham) of the carcass is different. Entire male pigs have approximately 500g more weight in the fore-end and 500g less weight in the ham compared with castrates. This difference in weight and lean meat percentage between the genders and three-parts was evaluated by computed tomography, using the same method as described by Olsen et al. [8]. If the carcasses are sold as three-parts, the higher meat percentage for entire males will be lost due to the higher value of the hams compared to the value of the fore-end.

Furthermore, the meat quality differs between genders. In one study, drip loss was higher in entire male pigs than in castrates [9], indicating a lower water holding capacity. Also the protein content differs between genders, being lower in entire male pigs than in females and castrates [10], which is important if the meat is used for further processing. For fresh meat consumption, not only is boar taint of importance, but tenderness is also, and a study has shown that tenderness is very important for consumer response even towards boar tainted meat [11]. Several studies have demonstrated a lower

tenderness in meat from entire male pigs compared with castrates [10-12]. However, even though these meat quality differences exist, they are difficult to value.

## 5. Processing of tainted meat

If rejection limits for boar taint are established, a certain number of the carcasses will be discarded and cannot be used for fresh meat consumption. The number depends on several factors: How restrictive are the sorting limits? How efficient is the primary production in reducing the concentration of skatole? How heavy are the pigs? Still, a considerable amount of the discarded meat must be used for other purposes to avoid food waste and consolidate the economics of production. Strategies for masking boar taint are, therefore, intensively investigated.

### 5.1. Smoking

Smoking is one of the most promising strategies for masking boar taint, and it has been widely discussed in the literature [13-17]. When discussing the masking effect of smoke, it is important to include a description of how intensive the smoked flavour is, and also how boar tainted the meat is. We have shown that smoking at 45°C for up to 60 min cannot fully mask boar taint in bacon from animals having a high concentration of skatole (0.6 µg/g in back fat) and androstenone (5.8 µg/g in back fat), while boar taint in sausages with the same level of skatole but lower in androstenone (2.4 µg/g in back fat) was fully masked after 60 minutes of smoking [13]. In contrast, another study showed that increased smoking time did not reduce boar taint, but in that study, the change in smoking time was only from 15 to 19 min, which might explain the lack of an effect [17]. Smoking can be regarded as an art, and using the same time/temperature/relative humidity in two different smoking chambers might result in a different intensity of smoked flavour. So even though smoking is a promising strategy for masking boar taint, both the smoking processing parameters and the intensity of boar taint must be taken into account. In practice, the strategy must be adapted to the individual product and smoking chamber.

### 5.2. Masking with herbs and spices

Several pork products and dishes are made using different spices, and the masking effects of these are of interest. Relatively few studies have looked into the masking effect of spices in a systematic way, although some studies exist using e.g. coriander and mace [17], oregano [18] and a spice mix for sausages [16]. We screened several different herbs and spices added to meat balls, to set up some general guidelines for choice of masking agent. The most important factor seems to be that the spice or herb must have a low odour threshold (and thereby a strong flavour) such as cinnamon, thyme and rosemary. On this basis, a spice mix was developed for pulled pork including cinnamon and paprika. This spice mix was effective in masking androstenone while only limited masking was seen for skatole. Therefore, if spices and herbs are going to be used to mask boar taint, it is important to include meat samples high in both skatole and androstenone in the investigations.

### 5.3. Serving meals not meat

In most consumer studies related to boar taint, the meat or meat products are served to the consumers in a neutral way. However, in practice, most meat and meat products are eaten in a meal context. Even when served in a tomato sauce, minced pork from entire male pigs (2.0 µg/g androstenone and 0.3 µg/g skatole in melted backfat) was more disliked than pork from female and castrate pigs [19]. Serving pork chops with potatoes, beans and gravy did not alter the acceptability score of boar tainted meat [20]. In contrast, we have seen a clear reduction of boar taint detection, when meat products were served in a meal context, both with consumers and with a trained panel (unpublished data). Serving ham in a ham and cheese toasted sandwich was especially effective, as no significant linear effect was seen of skatole or androstenone on boar taint, even though high levels of the compounds were evaluated (up to 9.0 µg/g androstenone and 0.54 µg/g skatole). The consumers did not differentiate between the ham from castrates or entire male pigs in ham and cheese sandwiches (Hall test) or toast

(HUT). A similar result was seen when the pulled pork described in section 5.2 was served in a pulled pork slider. Therefore, using meat from tainted carcasses as meat products in a complex serving in which the taint can be masked by the meal is recommended.

## 6. Monetary value for the slaughterhouse of entire male pig production

Calculating the monetary value of entire male pig production is difficult as several factors – specific for the individual slaughterhouse – must be taken into account. It is necessary to analyse scenarios when estimating the monetary value of pig production. In the following sections, the value of three different scenarios is calculated (Table 1). In all scenarios, market access and the logistics in handling the discarded carcasses are not taken into account. All pigs are analysed for skatole and androstenone using the method described in Section 3. This cost is set to €1.20 per sample. Danish average meat prices are used as well as the estimated value for degradation of the tainted meat, reduced tenderness and reduced protein content.

### 6.1. A few discarded carcasses sold as bulk production

In this scenario, only 2% of the carcasses are discarded. Taken from a Danish random sample of pigs, this would equate to a sorting limit of 0.25 µg/g skatole and no sorting on androstenone. No value is set for the lower tenderness or the change in protein content. The approved carcasses are sold as three-parts without further cutting or processing. In this scenario, the extra cost per entire male carcass has been estimated as €3.36 per carcass.

### 6.2. Sorting on both skatole and androstenone

In this scenario, the carcasses are sorted on a combination of skatole and androstenone. The rejection level for androstenone is high (5 µg/g), while it is 0.25 µg/g for skatole, resulting in 4.5% of carcasses being discarded. The carcasses are divided into four, splitting the middle into belly and loin, and in addition, the bones are sold as spareribs. This means that the high meat percentage in the entire male pigs gives a positive monetary value while the high number of discarded carcasses has a negative monetary value. A small amount (the loin from 40% of the carcasses) is sold as fresh meat in which the low tenderness has a negative value, while another small part (the loin of the ham from 60% of the carcasses) is sold for further processing in which the low protein content has a negative value. In this scenario, the cost has been estimated as €3.96 per carcass.

### 6.3. Low sorting limits on skatole and androstenone

In this scenario, the sorting limits are set to 0.25 µg/g skatole and 2 µg/g androstenone to reduce the risk of a negative experience by the consumer. This would amount to 18% of carcasses being discarded. In addition, 5% of carcasses are sold as fresh meat having a negative value due to reduced tenderness, while 24% are sold for meat products with a negative value of protein. The carcasses are split as in scenario 6.2. Due to the very high number of discarded carcasses, this scenario is very expensive, being approximately €9.80 per carcass.

**Table 1.** Three different scenarios for estimating the monetary value for the slaughterhouse of entire male production. The examples are calculated using Danish average cost and with estimated value degradation for the tainted meat as well as the value of tenderness and protein content. For further details, please contact the author.

	Scenario 1	Scenario 2	Scenario 3
Rejection limits			
skatole	0.25 µg/g	0.25 µg/g	0.25 µg/g
androstenone	no	5 µg/g	2 µg/g

% discarded carcasses because of boar taint	2%	4.5%	18%
Carcass	Sold as fore-end, middle and ham	Sold as fore-end and ham while the middle is loin with 8 mm fat and belly	Sold as fore-end and ham while the middle is loin with 8 mm fat and belly
Eating quality	Tenderness has no value	The loin from 40% of the carcasses is sold as fresh meat. Low tenderness gives a low price	The loin from 40% of the carcasses is sold as fresh meat. Low tenderness gives a low price
Protein content	Protein content has no value	From 60% of the carcasses, the loin or the ham will be sold to products in which the protein content has a value	From 60% of the carcasses, the loin or the ham will be sold to products in which the protein content has a value
Boar taint analysis	All samples from entire male pigs	All samples from entire male pigs	All samples from entire male pigs
Monetary value per entire male carcass	€3.36	€3.96	€9.80

## 7. General discussion

Changing to produce entire male pigs will have consequences for the slaughterhouse, not only related to boar taint and sorting of the carcasses. Some factors, such as a high meat percentage, can have a positive monetary value, while other factors, such as the percentage of discarded carcasses, a lower protein content and less tender meat can have a negative monetary value. The three examples given to estimate the monetary cost of production show how difficult it is to set a given value on entire male pig production. Each slaughterhouse must calculate the effect using their own prices, not only for the meat from approved carcasses (i.e. boar taint acceptable carcasses), but also according to how they expect the cost to be formed for the discarded carcasses. Results from this research should help the industry in such decision making.

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# The potential of condensed tannin-rich feedstuff to affect the nutritional and sensory qualities of ruminant-based products

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**Abstract.** Over the last decade, interest has increased when it comes to using temperate forage legumes containing condensed tannins (CT) for ruminants. The reason for this is that CT have been shown to benefit animal health and performance, as CT reduce parasitic burden by gastrointestinal nematodes, prevent bloat and reduce urinary nitrogen losses. Less is known about their impact on the quality of ruminant-based products. This short review discusses various quality issues which could, based on the current knowledge on the mode of action of CT, be positively affected by forages that are rich in CT. The main focus is the fatty acid composition, as well as the sensory traits of the meat, milk and cheese. The results presented here show the potential for two tanniferous forage legumes to positively affect the deposition of polyunsaturated fatty acids (PUFA), especially the *n*-3 fatty acids in meat, milk and cheese, and to improve the organoleptic quality of these products. From the two plants tested (birdsfoot trefoil and sainfoin), it is evident the CT effect in the digestive tract depends on various factors like the CT level, the chemical composition of the CT and whether the CT in the plant are available in a soluble form or bound to proteins or carbohydrates of the plants.

## 1. Issues with ruminant-based products

Worldwide, ruminant-based products are already an important part of the human diet, and the rate of consumption is expected to continue to rise in the years to come. However, especially in developed countries, their consumption has stagnated or even decreased. This is surprising from a nutritional point of view, as red meat contains high biological value proteins and important micronutrients, such as vitamin B, iron (both free iron and haem iron) and zinc. Similarly, milk and dairy products are rich sources of protein, calcium and vitamins A and D. The responsibility for the stagnation or decline in consumption can be attributed to, among other things, increasing skepticism towards modern livestock husbandry practices, which affects consumption habits, and the bad reputation of livestock products with respect to health. This public perception is partly based on undifferentiated interpretations of scientific reports. For instance, it has been established that processing meat through curing, smoking, or cooking can result in the formation of known or suspected carcinogens, including N-nitroso-compounds, polycyclic aromatic hydrocarbons and heterocyclic aromatic amines [1]. The Working Group of the International Agency for Research on Cancer found that existing epidemiologic data is sufficient to classify processed meat as carcinogenic [1]. However, the same authors state that there is limited evidence for the carcinogenicity of unprocessed red meat, which is in line with the outcome of the systematic review of experimental results performed by Turner et al. [2]. These authors found no evidence of a mechanistic link between the intake of red meat as part of a healthy dietary pattern and



the risk of colorectal cancer. Similarly, ruminant fat is portrayed as a negative component of ruminant-based products, largely because it is a rich source of cholesterol and saturated fatty acids (SFA). The rationale behind the negative perception is that compared to dietary monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), dietary cholesterol and SFA raise risk factors for cardiovascular disease, such as the total serum cholesterol and the ratio of low-density lipoprotein to high-density lipoprotein cholesterol [3]. However, Kratz et al. [4] concluded that no evidence has been observed which supports the hypothesis that dairy fat contributes to any cardiometabolic risk.

The odor and flavor of food are crucial traits that strongly impact consumers' acceptance and willingness to purchase. These qualities are affected by an animal's diet, as the degradation and oxidation of feed components can lead to the formation and deposition of lipophilic odorant molecules in the milk and in the adipose tissue, the inter- and intra-muscular fat. Consequently, the presence of off-odor and off-flavor is dependent on the fat content of the products and can, thus, be a greater issue in high-fat products like butter, cream or full-fat cheese. The deviant flavor which consumers primarily perceive if they have become accustomed to the flavor of meat from grain-finished animals is the pastoral or dairy flavor. This flavor can be found in the dairy products or meat from pasture-grazing ruminants. Pastoral flavor summarizes attributes like *grassy*, *milky*, *animal-like*, *barnyard* and *fecal*, and have been related to the presence of skatole and indole [5, 6], both of which originate from the ruminal tryptophan catabolism, which produces indole and indole acetic acid.

## 2. Fate of dietary PUFA

Despite the fact that grass and hay, the typical dietary components of ruminant rations, contain high quantities of unsaturated fat (more than 70% PUFA, primarily of the *n*-3 family), fats of ruminant-based products are mainly saturated. The primary reason is that after lipolysis by plant and microbial lipases, up to 90% of free oleic (18:1*n*-9), linoleic (18:2*n*-6) and linolenic acids (18:3*n*-3) in the rumen undergo ruminal biohydrogenation [7], which results in the accumulation of stearic acids (18:0) and trans-fatty acids (e.g. vaccenic acid [*t*11-18:1]) [8] in the small intestine. After absorption, a part of these fatty acids are converted in the mammary gland, muscle and adipose tissue by  $\Delta$ 9-desaturase to palmitoleic acid (16:1*n*-7), oleic acid or rumenic acid (*c*9,*t*11-18:2 [Conjugated Linoleic Acid], [8]). Since neither linoleic nor linolenic acids can be synthesized by mammals, linoleic and linolenic acids need to be ingested through the mammal's diet. Consequently, to naturally enrich ruminant-based products with PUFA, the extent of ruminal biohydrogenation needs to be reduced. To do this, approaches have been tested that have proven successful, such as coating the dietary fat source and/or using *n*-6 and/or *n*-3 PUFA rich ingredients [9]. These strategies targeted the dietary PUFA source, whereas an alternative approach could be inhibiting the microbial population responsible for ruminal biohydrogenation (e.g. *Butyrivibrio* spp., *Propionibacterium acnes*, *Megasphaera elsdenii* [10]). The latter method would allow the exploitation of the natural PUFA sources, namely, grass and hay, without necessarily requesting additional sources of fat.

## 3. Bioactive compounds of plants

The aforementioned quality constraints of ruminant-based products require remedying, and the bioactive compounds, also called secondary metabolites, found in forage legumes could provide such a solution. The secondary metabolites in plants are allelochemicals [11] and include compounds which are not necessary for vital functions like plant reproduction or growth. There are three main families of bioactive compounds: *alkaloids*, *terpenoids* and *phenolic compounds*. Among the wide class of phenolic compounds rich in hydroxyl and in phenolic groups, condensed tannins (CT) are of particular interest for the quality of ruminant-based food.

### 3.1. Condensed tannins

Condensed tannins, also called proanthocyanidins, are oligomers (2 to 10 monomers) or polymers (> 10 monomers) of flavan-3-ol units; their name is based on how proanthocyanidins, when treated with acidic alcohol, will degrade to anthocyanidins, the pink-purple pigments responsible for the color of

flowers. CT are not susceptible to anaerobic enzyme degradation [12], and it has been assumed until now that their structure is only marginally altered in the digestive tract [13]. Based on the position of the –OH and –H groups, several flavan-3-ol units give rise to classes of polymers such as procyanidins, prodelphinidins, profisetinidins and prorobinetinidins [11]. When found in plants, CT can be present as a mixture of these classes. For instance, CT from quebracho (*Schinopsis lorentzii*) are polymers of profisetinidins and prorobinetinidins, while CT from temperate forage legumes are polymers of procyanidins and prodelphinidins. Furthermore, within species of forage legumes, procyanidins are found in greater abundance in birdsfoot trefoil, whereas prodelphinidins constitute the main polymer class in sainfoin [11].

### 3.2. Interaction of CT with other molecules

Owing to their hydroxyl and phenolic groups, CT can establish different types of interactions with proteins, lipids, carbohydrates and metal ions [14]. The interaction between CT and other molecules is based on weak linkages such as hydrogen bonding or hydrophobic interactions [15]. The weak linkages are reversible and can dissociate depending on physico-chemical conditions such as temperature and pH. In the context of this review, the ability of CT to precipitate protein and lipids under certain physico-chemical conditions is of interest. The most abundant water-soluble protein of fresh forages, dried herbages, silages or hay is RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase). A pH between 3.5 and 7, like in the rumen, is generally favorable for generating the CT-RuBisCO complex, whereas at a lower pH, like in the abomasum, or at higher pH, this complex becomes dissociated and RuBisCO is released [16, 17]. In addition, the affinity to form CT-protein complexes is affected by the size of the polymers and the procyanidin-to-prodelphinidin ratio. In general, as the polymer length increases and the concentration of prodelphinidin becomes greater, the protein precipitation properties of CT also increase (reviewed by Girard et al. [13]).

### 3.3. Biological properties and presumed mode of action of CT

The mechanism of CT action in the digestive tract has not been fully unraveled. In view of the previously described interaction of CT with other molecules, in ruminant nutrition, the following mechanisms have been proposed:

- 1) Binding and dissociation, based on the surrounding pH milieu of proteins, carbohydrates and lipids, and by that protecting the substrate (e.g. nutrients from the diet) from microbial degradation in some parts of the digestive tract (e.g. in the rumen but not the small intestine).
- 2) CT change the conformation and the microbial enzyme activities and by chelation the availability of vital ions of rumen microorganisms.

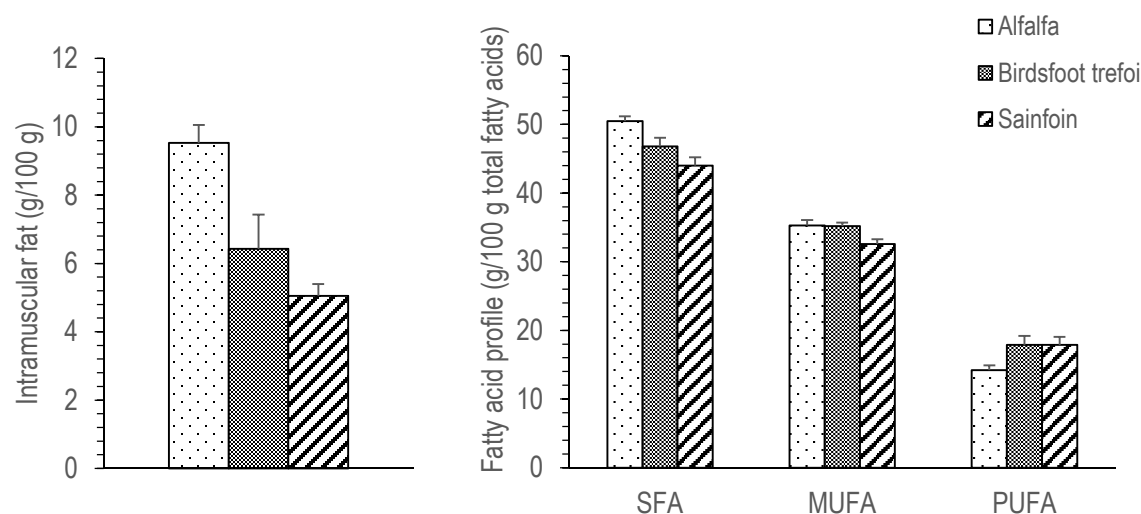
These effects in the digestive tract could ultimately be relevant for the final quality of ruminant-based products.

## 4. From theory to practice – to what extent do CT affect ruminant-based products?

The following section will summarize the results of experiments performed during the European-funded Marie Curie Initial Training Network project named LegumePlus (<http://legumeplus.eu>). Detailed information of the studies is available online [18, 19]. Two major CT-containing forage legumes were used in the studies: sainfoin and birdsfoot trefoil.

### 4.1. Effects of tanniferous forage legumes on quality traits of lamb meat





**Figure 1.** The intramuscular fat content and fatty acid profiles of the intramuscular fat of the longissimus thoracis muscle of lambs fed alfalfa, birdsfoot trefoil or sainfoin silages.



**Figure 2.** PUFA levels of the intramuscular fat of the longissimus thoracis muscle of lambs fed alfalfa, birdsfoot trefoil or sainfoin silages.

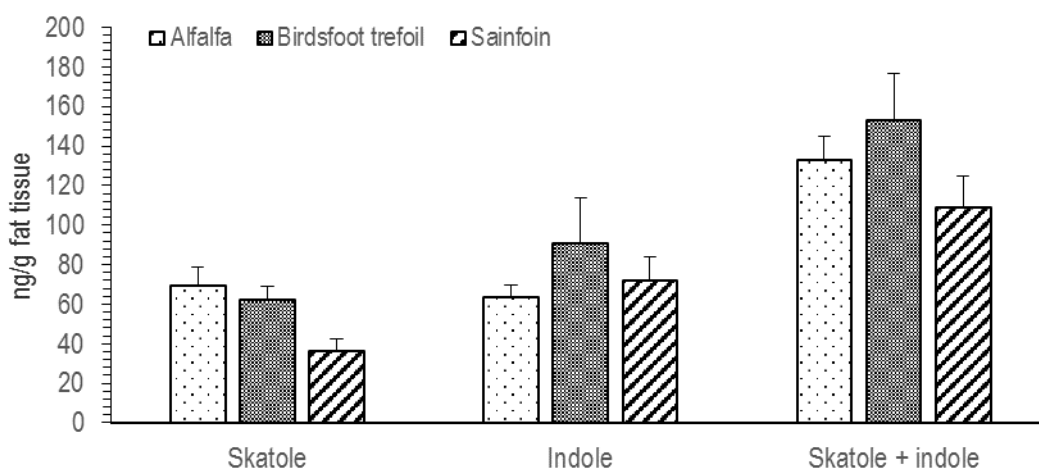
As previously mentioned, low PUFA levels and the pastoral flavor can be determinant traits for consumer acceptance of ruminant-based products. To assess the potential of CT to alter these traits, an experiment was performed with growing lambs. The animals weighing 21 kg were offered either birdsfoot trefoil, sainfoin or alfalfa silage *ad libitum* for, on average, 127 d. The sainfoin contained five times more CT than the birdsfoot trefoil (10 vs 21 g/kg dry matter). Average daily gain, hot carcass weight and dressing percentage of lambs fed birdsfoot trefoil and sainfoin were lower compared to lambs fed alfalfa (56 and 58 vs 102 g/d; 11.4 and 12.0 vs 15.6 kg; 39.9 and 41.1 vs 45.5%). The diets showed no effects with respect to meat quality traits of water holding capacity, meat color or shear force. There are contradictory results regarding the impact of diets containing elevated CT levels on animal growth, feed intake and feed palatability [20, 21]. Lower palatability has been related to the interaction between CT and salivary proteins such as proline, which creates an impression of astringency, thereby reducing voluntary feed intake [22]. In addition, greater concentrations of CT impair the degradation of ruminal nutrients (desired in the case of the protein).

This may explain why growth performance and consequently carcass weight of both, birdsfoot trefoil and sainfoin lambs, were inferior to alfalfa lambs.

In accordance with the lower growth rate, the intramuscular fat content was lower in lambs of the birdsfoot trefoil and sainfoin groups compared to the alfalfa group (**Figure** ). The intramuscular fat of lambs fed birdsfoot trefoil and sainfoin contained less SFA and more PUFA than those fed alfalfa (**Figure** ), resulting in a PUFA-to-SFA ratio of 0.38, 0.53 and 0.28, respectively. In addition, compared to alfalfa, the tanniferous plants increased the relative amounts of the long-chain fatty acid homologues of the *n*-3 and *n*-6 families by 117 and 196%, respectively (Figure 2). Interestingly, the level of long chain PUFA, such as arachidonic acid (20:4*n*-6), eicosapentaenoic acid (20:5*n*-3) and docosapentaenoic acid (22:5*n*-3), were up to 43% greater in the intramuscular fat of lambs fed sainfoin compared to those fed birdsfoot trefoil (Figure 2). This finding suggests that the relative levels of the elongation products of linoleic acid (18:3*n*-3) in the intramuscular fat could be dose-dependent in terms of the CT intake (the CT level was greater in sainfoin than in birdsfoot trefoil and feed intake was similar) and/or chemical structure-dependent in terms of the CT molecules (sainfoin contains more prodelphinidins and fewer procyanidins compared to birdsfoot trefoil). As the linoleic acid intake per lamb was estimated to amount to only 3 g for lambs fed birdsfoot trefoil and sainfoin compared to 5 g for the lambs fed alfalfa, it can be assumed that the linoleic acid transfer rate was greater as a result of a reduced ruminal biohydrogenation rate in the lambs that were fed CT-rich legumes. The same is valid for linolenic acid.

The concentration of skatole and indole in the perirenal fat was determined using high-performance liquid chromatography [23]. The perirenal fat of the sainfoin lambs, but not of the birdsfoot trefoil lambs, contained almost double the skatole concentration than that of the lambs fed alfalfa (

Figure 3). In contrast, neither the concentration of indole nor the sum of indolic compounds were affected by the dietary treatments. It is well established that decarboxylation of tryptophan in the rumen by *Lactobacillus* sp., *Clostridium scatologenes*, and *Clostridium drakei* [24] forms indole and indole acetic acid, which is further decarboxylated to indole-3-methyl (skatole) [25]. Indole and skatole pass the rumen wall, and, regulated by specific CYP450 isoenzymes, undergo hepatic clearance [26]. However, depending on the amount of skatole and indole produced in the rumen, degradation by specific cytochrome P450 isoenzymes can be incomplete, resulting in an accumulation of both substances in the adipose tissue. Studies performed *in vitro* found that the inhibitory effect of CT targeted the transformation of indole acetic acid to skatole more than it targeted the formation of indole itself [27], which is in line with our findings *in vivo*. The lack of birdsfoot trefoil's effect on skatole and indole was surprising, as studies in New Zealand have reported lower concentrations of skatole and indole in the body fat of lambs that graze birdsfoot trefoil swards [6]. As for the fatty acid



**Figure 3.** Skatole and indole levels determined in perirenal fat of lambs fed alfalfa, birdsfoot trefoil or

metabolism, it appears that the quantity and/or the chemical structure of a plant's CT determines its effect on the production of skatole and indole.

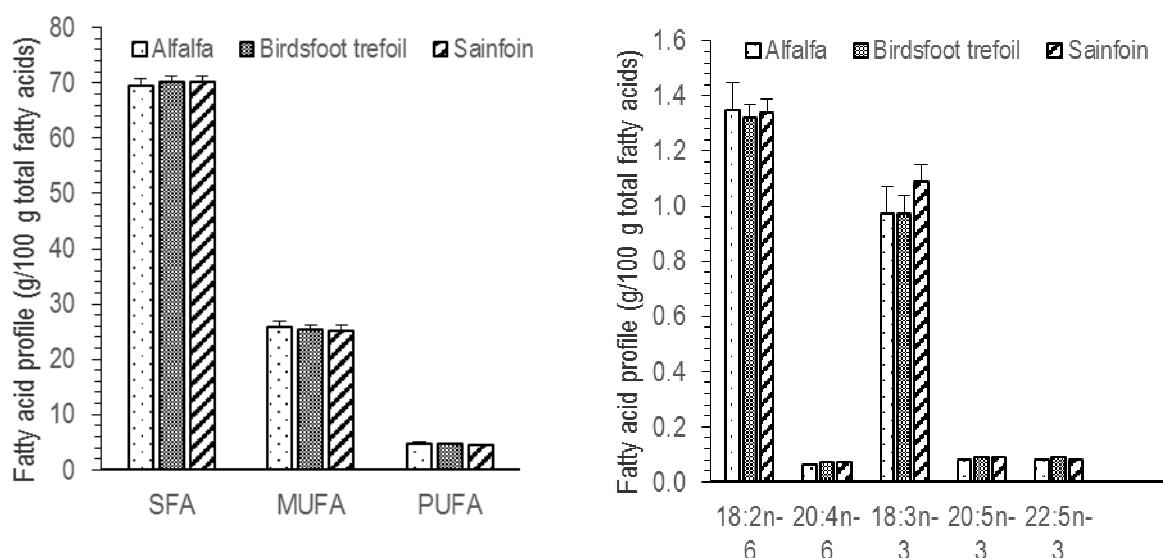
To assess the impact of CT-containing legumes on the pastoral flavor of lamb meat, sensory analysis was conducted with a trained panel using known descriptors to judge the flavor of the lamb meat. The intensity of the descriptors *grassy*, *milky*, *sweet* and *sour* for the cooked lamb meat was judged to be similar among the three treatment groups. Of all three treatment groups, meat from the lambs of the sainfoin group had the weakest 'sheepy' odor and flavor. These findings are in line with the low skatole level, since skatole is believed to play a key role in the development of pastoral flavor [5].

In conclusion, the present data has demonstrated the potential of ensiled legume species with elevated CT contents to modify the nutritional value and sensory properties of lamb meat. Feeding lambs solely these legumes increased the relative content of long chain *n*-3 fatty acids, but the growth performance and intramuscular fat deposition rate were impaired. Among the CT plants tested, sainfoin was more efficient than birdsfoot trefoil at reducing the ruminal biohydrogenation of dietary PUFA, reducing skatole concentrations, and, to some extent, reducing the pastoral off-flavor.

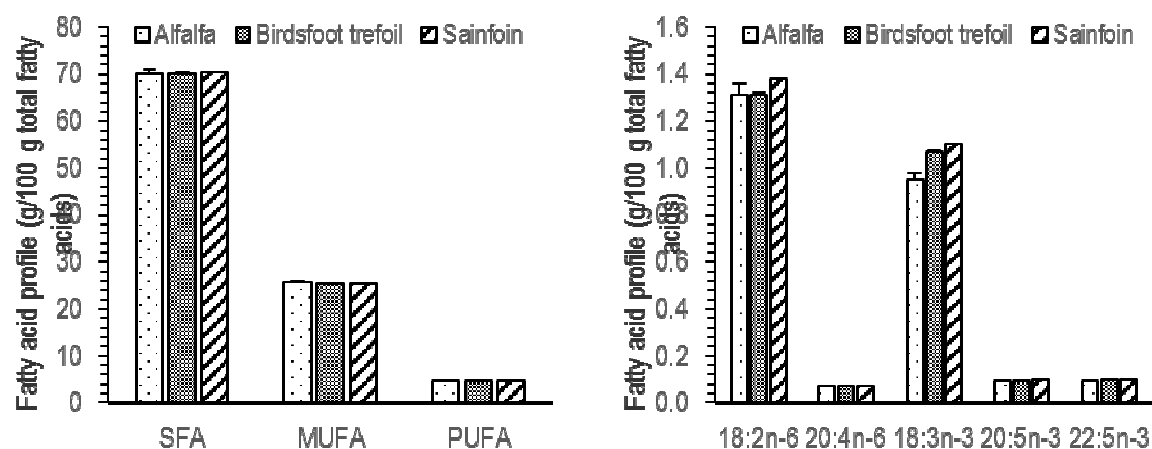
#### 4.2. *Effects of tanniferous forage legumes on the quality of milk and Gruyère-type cheese*

As previously reported, due to ruminal processes, the PUFA level in milk and dairy products does not reflect the dietary PUFA intake. As proven in the lamb study, PUFA biohydrogenation is limited by CT, resulting in elevated levels of long-chain PUFA. Therefore, a feeding experiment lasting 52 d was conducted with 18 Holstein cows to determine whether diets containing CT from birdsfoot trefoil and sainfoin increase the PUFA, especially the *n*-3 fatty acid content, in the milk and cheese, without negatively affecting their physico-chemical and sensorial properties. Cows were assigned to the three treatment groups. The basal diet was composed of hay, corn silage, Extrulin (a rich source of linolenic acid), concentrate and alfalfa in a ratio of 45:25:5:7:18. For the CT groups, the portion of alfalfa pellets was replaced by either birdsfoot trefoil pellets (CT = 3%) or sainfoin pellets (CT = 19%). The milk was collected on three consecutive days. The milk from each individual cow from each day was analyzed for milk gross composition and fatty acid profile. The milk of all six cows from each day of treatment was combined and processed into a Gruyère-type cheese. A trained panel assessed the sensory quality of the raw milk and cheese using discriminative and descriptive tests [28].

The dry matter intake, milk yield and milk gross chemical composition did not differ among treatment groups. Except for small changes in the proportions of some individual fatty acids, the



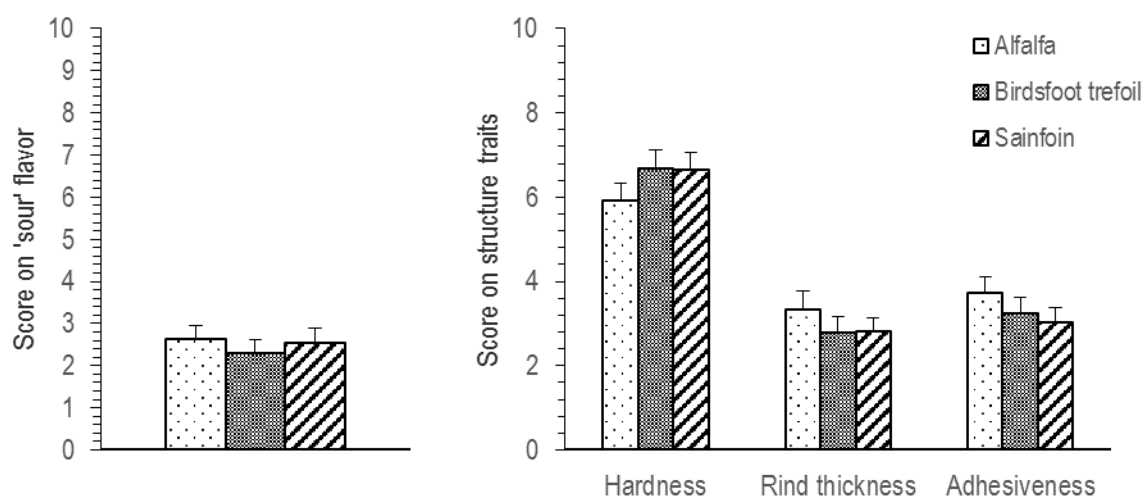
**Figure 4.** Fatty acid profiles of the milk from cows fed standard diets supplemented with either alfalfa, birdsfoot trefoil or sainfoin.



**Figure 5.** Fatty acid profiles of cheeses produced from the milk of cows fed standard diets supplemented with either alfalfa, birdsfoot trefoil or sainfoin.

proportions of total SFA, MUFA, PUFA, *n*-3 and *n*-6 fatty acids were not affected by the dietary treatments (**Figure** ). Similar to the milk fat composition, the relative proportions of the main fatty acid groups in the cheese were not significantly affected by the diets (**Figure** ). However, compared to the alfalfa group, the sainfoin pellets slightly but significantly elevated the linoleic and linolenic acid levels as well as the proportions of two of its elongation products, eicosapentaenoic and docosapentaenoic acid, in the cheese. The treatment effects observed in the cheese but not in the milk can be explained by the greater variability in the fatty acid profile of the milk from the six individual cows per treatment compared to the fatty acid profile of the cheeses produced from a mix of the milk from those individual cows. For instance, after feeding the sainfoin pellets, the linolenic acid level increased by 17% in both the milk and the cheese. The increase is statistically significant only in the cheese for which the variability expressed as standard error of the mean was 12 times lower than in the milk. As with the sheep, there were several indications to suggest that dairy cows also experience ruminal biohydrogenation inhibition via CT-rich forage legumes (with sainfoin having a greater effect than birdsfoot trefoil): first, the increase in the level of the *n*-3 fatty acid family; second, the elevated levels of oleic acid; third, the lower proportion of stearic acid (the terminal product of ruminal biohydrogenation). As the effects of birdsfoot trefoil were not as great as the effects of sainfoin, and as birdsfoot trefoil had markedly lower CT content than sainfoin, it is implied that a certain CT level is needed for the inhibition of PUFA biohydrogenation. However, the present data does not allow the establishment of a distinct threshold value.

Regarding sensory evaluation, panelists did not detect any differences in either the milk odor, or the intensity of flavor in the cheese between the birdsfoot trefoil and sainfoin groups compared to the alfalfa group. These findings corroborate with the lack of difference in the gross chemical composition of the milk and the cheese, and the changes occurring in the fatty acid profile of the milk and cheese were apparently too small to affect the sensory assessment. With respect to texture, cheeses from the birdsfoot trefoil and sainfoin groups were judged harder and tended to be less adhesive to the palate compared to those cheeses from the alfalfa group (**Figure 6**). In addition, birdsfoot trefoil and sainfoin cheeses had less rind. The firmness of cheese depends on the palmitic acid (16:0) and oleic acid content in the milk fat, with palmitic and oleic acid levels correlating to the hardness and the softness of cheese, respectively [29, 30]. An increase in the moisture content of the cheese has been correlated to an increase in cheese adhesiveness [31]. However, neither the levels of palmitic or oleic acids nor the moisture contents were responsible for the observed structural changes, as these traits were similar



**Figure 6.** Sensory evaluation grades for the flavor and structure traits of the cheeses aged for 8 months, produced using the milk from cows fed standard diets supplemented with either alfalfa, birdsfoot trefoil or sainfoin. The scale ranges from 0 (low intensity/thin) to 10 (high intensity/thick).

among the cheeses produced from the three experimental treatments.

In conclusion, the results of the present study show that with a CT-rich forage legume like sainfoin, resulting in a diet containing 3% total CT, the level of linolenic acid and of long-chain *n*-3 fatty acids can be elevated in dairy products. This confirms that elevated dietary CT intake can increase the proportions of some beneficial PUFA in both milk and cheese. However, the effects of birdsfoot trefoil were not comparable to the effects of sainfoin, which supports the hypothesis that the effects depend on the plant species and in this case, most likely to the difference in the CT content. The present study has also demonstrated that the inclusion of 18% sainfoin in dairy cow diets makes it possible to produce cheese with only a few distinguishable sensory changes, and these descriptors concerned structure more than flavor. This largely disproves that CT negatively affects the odor and flavor in the tested dietary situations. Another important finding which promotes the use of sainfoin in the diet of dairy cows was that sainfoin can replace a high quality legume like alfalfa without adverse side effects on feed intake and milk yield.

## 5. Overall conclusion

The objective for using CT-rich plants in ruminants was to determine the efficacy of CT to increase the content of PUFA, especially of *n*-3 fatty acids, and to decrease the content of SFA in the ruminant-based products by reducing the ruminal biohydrogenation of dietary PUFA. From the two CT-rich forage legumes tested, sainfoin was always the most effective, but the effect was greater in meat than in milk. Several reasons could determine these differences, from species (lamb vs cow), to forage form (silage used with lambs vs dehydrated pellets used with dairy cows), to CT levels and feeding duration. As we have shown, the type of forage form can affect the structure of CT [32]. In the silage, the main proportion of CT is bound to plant proteins, while in the pellets, CT are in a soluble form. Until now, it has been unclear to what extent the form of binding impacts the efficacy of the CT action. Furthermore, whereas the lambs were fed solely CT-rich silages for over 100 d, the dairy cows consumed the CT-rich plants as only a portion (18%) of the whole diet, and it was offered for a much shorter period (30 d). This obviously raises the question of the level of CT to include in rations to show a significantly positive effect, as well as the duration for which CT should be offered to the animals before a maximal effect can be seen.

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# Suckling lamb meat: A smart and sustainable food combining tradition and innovation

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**Abstract.** Meat from milk-fed lambs is one of the most relevant products in the traditional dairy sheep industry. This product is the meat from suckling lambs fed maternal milk in flocks raised almost only on pasture. The aim of this paper is to briefly summarise current knowledge of the qualitative traits of suckling lamb meat, with special attention paid to the lipid fraction. We report the main scientific data for suckling lamb meat that contributes to its alleged properties as a functional and environmentally sustainable food. Furthermore, reference is made to how innovative chill/freeze technologies can be profitable, enlarging consumption of this meat in the dynamic global markets.

## 1. Introduction

The sheep industry is one of the most ancient and traditional worldwide farming systems since the domestication of sheep occurred about 11,500 years ago [1]. In comparison with other livestock species, sheep are characterised by their ability to efficiently exploit pastures, even when these are scarce or inadequate for large ruminants. Modern dairy sheep breeds are high performing in terms of milk yield when their diet is properly balanced with their genetic potential.

The main relevant income of dairy sheep flocks is from their milk, which is mostly processed into dairy products, apart from the milk suckled by lambs. However, in dairy sheep systems, meat production from suckling lambs can be found worldwide [2].

Livestock systems with ruminants fed on pasture do not compete directly with humans because the lands used are usually unsuitable for other crop-foods, and this diversity contributes to food security. In fact, ruminants play a fundamental role in food chain sustainability, since they are able to convert renewable resources from rangeland like pastures into food edible for humans, like milk and meat. The efficiency of ruminant livestock in converting feeds into edible energy and protein for humans is extremely dependent on the farming system. Suckling lambs, finished to an approximate body weight of 10 kg at 30-35 days of age, are a traditional product in many dairy sheep farms in Mediterranean regions. Meat production from suckling lambs in grazing flocks is a food production process whereby grass is converted into meat, milk being an intermediate product. In the first weeks of life, lambs do not have a fully functional forestomach, and therefore, their digestive physiology behaves as in monogastric animals. This means the gastrointestinal tract of suckling lambs does not host microbial fermentation processes, which generate the by-product methane that contributes to greenhouse gas emissions. The wide diversity of sheep breeds and production systems are the main variables affecting the large variability of suckling lamb carcasses on the markets [2]. Among this variability, a singular product is





obtained when lambs are nourished solely on milk from ewes fed on pasture during lactation. Suckling lamb meat, as well as being a traditional food in the Mediterranean area, has some unique nutritional characteristics that make it particularly suitable for childhood nutrition [3] [4].

This short review summarises the nutritional characteristics of suckling lamb meat produced by dairy sheep reared in pasture-based production systems. Moreover, it presents the advantageous nutritional composition of key nutrients such as the lipid fraction, with particular reference to fatty acids (FAs). This affects the degree of acceptance of this meat by consumers, who are also interested in the ecological sustainability of the product.

## 2. Suckling lamb – “smart” meat

Innovation in the food industry is strongly aimed at improving the nutritional profiles of meat and meat products to produce those with healthier properties for consumers.

Many studies have demonstrated that meat and meat products can be considered as functional foods, as sources of indispensable nutrients with proven health benefits for humans [5]. Meat plays an important role in the human diet by being a good source of high quality protein as well as beneficial FAs and a range of micronutrients, like vitamins (especially B12) and minerals (especially iron) with a high degree of bioavailability. However, the breeding system has an important effect on the nutritional characteristics of meat. The presence of pasture in the diets strongly enhances the nutritional quality of intramuscular fat compared to dry forage diets. Fresh grass in the diet of lactating ewes is useful to increase the presence of biologically active compounds in milk such as unsaturated FAs with positive health properties [6] [7] [8] [9], particularly vaccenic acid (VA; C18:1 t11), rumenic acid (RA; c9,t11 conjugated linoleic acid), and  $\alpha$ -linolenic acid (ALA; C18:3n-3).

These nutraceutical properties of ovine milk can contribute to enriching the health value of meat from lambs suckling maternal milk and improve the meat's value as functional food. A comparison of the FA profile of fresh meat (FM) from suckling lambs with commercial infant foods based on lamb meat, such as homogenised and lyophilised meats, was reported [4]. FM contained greater contents of ALA (1.5-fold higher) and its elongation products (eicosapentaenoic acid, 20:5n-3, EPA; and docosahexaenoic acid, 22:6n-3, DHA), and arachidonic acid (C20:4n-6, ARA) derived directly from the linoleic acid (C18:2n-6, LA), than the infant foods [4]. Those results documented that suckling lamb meat is also an interesting food source of some long-chain polyunsaturated FAs that are essential for proper development of foetal brain and eyes and maintenance of neural and visual system throughout life [10] [11]. Since the potential lower allergenicity of lamb meat compared to other red meat sources is clear [12] [13], its enrichment with PUFA<sub>n</sub>-3 could be of nutritional interest, especially in weaning diets for children.

### 2.1. Milk suckled by lambs from pasture-fed ewes

Feeding systems of lactating ewes are an effective strategy to improve lamb meat quality in terms of fat content and composition. The composition of ewe milk strongly affects the growth performance and meat characteristics such as the FA composition of suckling lambs.

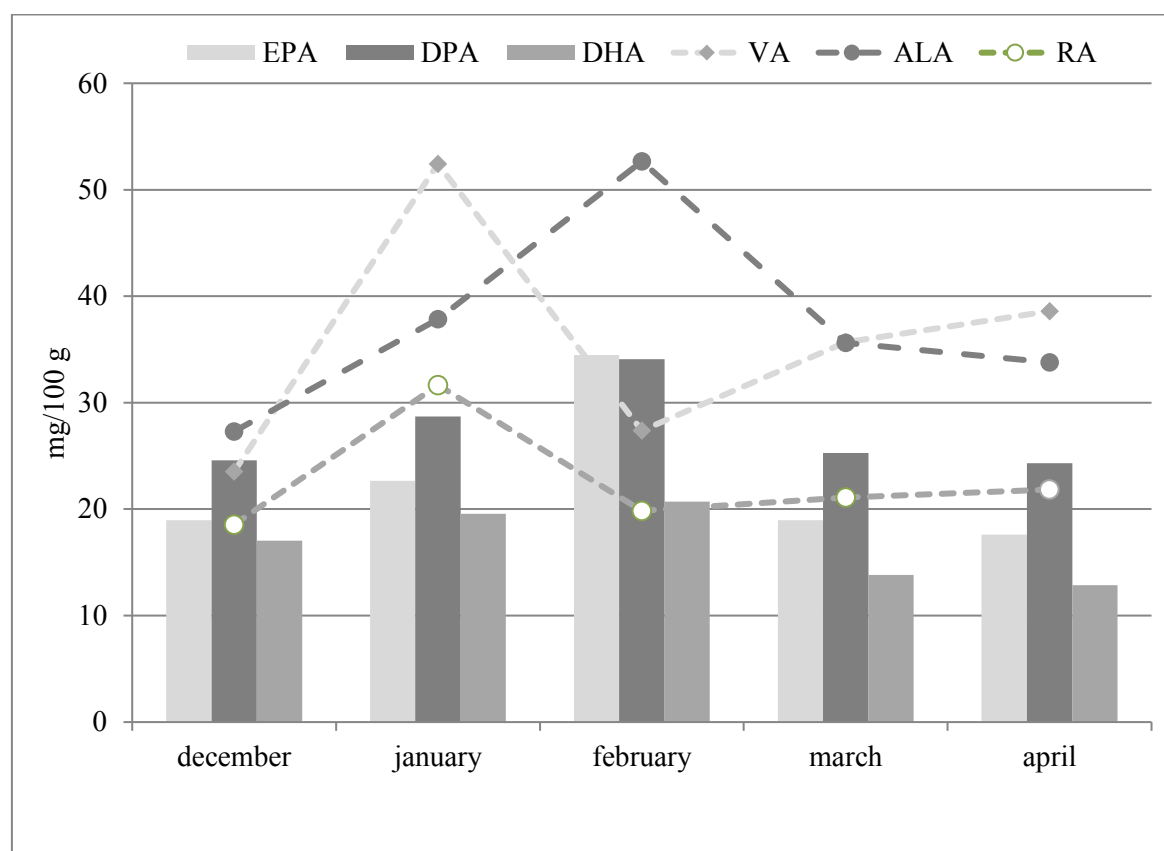
During lactation of dairy sheep fed on pastures, the FA profile in milk is affected by the variation of diet, i.e. the progressive variation of FAs in maturing grass [14]. In pastures, the main FA is ALA [15] that decreases as grass matures and in temperate climates (such as in Europe and North America) reaches the highest levels in pastures during the winter-spring months [16]. Feeding lactating ewes on pastures is an advisable alternative to increase the contents of VA, RA and long-chain n-3 polyunsaturated FAs (PUFA<sub>n</sub>-3) in milk, since fresh grass has a higher concentration of ALA than diets containing concentrates or dried forages [17]. Actually, the forage species and their phenological phase also affect the FA profile in ovine milk, since the FA contents in different pasture species are highly variable [18] [19]. In the traditional breeding systems in Mediterranean areas, the lambing period occurs in late autumn to early winter to allow the ewes to exploit the seasonal availability of the natural pastures at their best. This allows the lambs to start suckling at the very time when levels of VA, RA and PUFA<sub>n</sub>-3 in maternal milk are the highest. Moreover, the presence of pasture in the diet of the ewes is also the

main source of other compounds with antioxidant activities, such as vitamin E and carotenoids that are carried over from milk to meat [20].

## 2.2. Lamb meat affected by ewe milk

The FA profile of suckling lambs reared on maternal milk reflects the FA profile of the suckled milk. In this phase, lambs being functionally non-ruminants, the ruminal biohydrogenation of the milk FAs does not occur before they are absorbed from the intestine. The rate of *de novo* fat synthesis in suckling lambs is very low, and most of the deposited FAs are derived directly from their diets. The FA source in the diet of suckling lambs has been shown to strongly affect the FA profile of different lamb tissues in different manners [21]. Moreover, the FA profile of suckling lamb meat could be different when lactating ewes graze on pasture or are fed dry diets. In particular, suckling lamb meat reared from ewes fed on pasture has greater percentages of RA, ALA and PUFA<sub>n</sub>-3 and a lower percentage of PUFA<sub>n</sub>-6 [22]. However, the composition of suckling lamb meat is affected by the feeding system of the mother rather than the management system (indoor vs outdoor) of lambs [23]. In some cases, when dairy sheep farmers are focused on achieving the best economic returns for the marketable milk yielded, lambs are removed from their mothers and are raised with artificial milk. In these conditions, milk replacer adversely affected the nutritional value of the FA profile of lamb meat [24] [25]. The relationships between the proportions of FAs in milk and meat differed markedly for the different FAs. Relationships between the FAs of ewe milk and those of the suckling lamb meat changed for each FA [22]. In particular, the percentages of total C18:1 and RA in meat mirrored those in milk, with high correlations, whereas the PUFA<sub>n</sub>3 in meat is higher than in milk, but is not correlated. In meat, this could be due to *de novo* synthesis from ALA of long-chain PUFAs such as EPA and DHA that are commonly present in muscle tissue, whereas they are not regularly detected in milk. Moreover, the essential FAs in milk could be used to different extents by different tissues in growing lambs, e.g. the brain and the nervous system.

A survey was conducted by our research group to evaluate seasonal variation of the FA composition of meat from Sarda suckling lambs reared in Sardinia (Italy) and slaughtered from December to April. In Sardinia, as in other Mediterranean countries, the main lambing season is during the end of autumn and the early part of winter. During this time, the ewes are fed mainly on pasture and receive a low amount of dry feeds (concentrate and hay). In fact, the weather conditions are favourable for the growth and availability of pasture. In Figure 1, we report the amount of FA of nutritional interest as mg/100 g of edible muscle during December to April. The ALA, CLA and long chain PUFA<sub>n</sub>-3, i.e. EPA, DPA (22:5 n-3) and DHA, evidenced only slight variation during these months. Because Sardinian suckling lambs are fed almost exclusively milk from their mothers, variations in meat FA profile can be attributed mainly to changes in the diet of their dams, supported by the ALA levels tending to reduce in the pasture over time and, therefore, in milk of the lactating ewes.



**Figure 1.** Fatty acids of nutritional interest (as mg/100 g of fresh meat) in the intramuscular fat of *Femoral biceps* from Sarda suckling lambs in different slaughter months (data from [26])

### 3. Suckling lamb as sustainable meat

The dairy sheep system, also in terms of suckling lamb meat, impacts on production and consumption of agricultural products. Life cycle assessment (LCA) is a methodology to account for resource use and emissions throughout the full life cycle of each product and evaluate its environmental sustainability. Application of LCA to livestock production systems is a relatively new area of research [27]. Several studies have been published on dairy and beef cattle and on meat sheep. Estimates of the carbon footprint (CF) of lamb meat production were carried out in Europe and Oceania, highlighting the relevance of sheep production systems in these two areas. Among studies, the average CF of lamb meat varied from 5.0 [28] (Australia with sub-clover systems) to 25.9 [29] kg of CO<sub>2</sub>-eq/kg of LW (live weight) lamb meat, depending on the production systems and the estimation method used. Looking at published values, within-study variation can be much higher, and Benoit and Dakpo (2012) [30], surveying 1180 farms, obtained values ranging from 12 to 82 kg of CO<sub>2</sub>-eq/kg of LW lamb meat. Studies mainly focused on lamb produced from meat breeds, whereas no specific values indicated CF estimates for suckling lambs from dairy breeds. Table 1 shows estimates of CF emissions of suckling lambs produced in Sardinia within mixed production systems. It assumes that milk used for lamb feeding, enteric and manure emissions are the main emission hotspots. In particular, the emissions generated in the milk production processes are the largest emission source, and are mainly related to the feed efficiency and the production level of the flock in recent estimates [31] [32] [33]. Flocks with higher production level per head are able to produce milk with lower CF, which also results in lower emissions for the suckling lamb meat produced. Estimated values ranged from 7.7 to 16.5 kg of CO<sub>2</sub> eq. per kg of LW for 300 and 100 kg/year of milk per head produced by the mothers, respectively. If lambs are separated from the

dams at birth and fed milk replacer until slaughter at 30 days, the CF of the lamb meat is almost doubled (32.5 kg of CO<sub>2</sub> eq. per kg of lamb LW). This is due to the higher emissions of the milk replacer compared to the sheep milk, even if from an economics point of view, the artificial feeding could be more convenient due to the low price of milk replacer (0.25 €/kg of solids) in comparison to the sheep milk (5.5 €/kg of solids). In summary, suckling lamb production from dairy breeds can be considered highly comparable in terms of sustainability, measured as global warming potential, to the lamb meat produced from meat breeds.

**Table 1.** Carbon footprint estimates of meat production from suckling lambs in mixed production systems in Sardinia. The estimations assume that milk used for lamb feeding and enteric and manure emissions are the main emission sources.

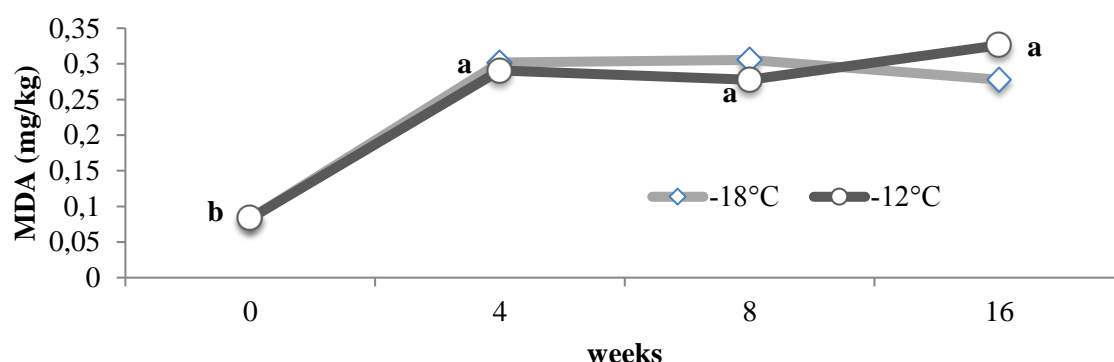
Lamb type	Suckling lamb from dairy breeds raised in mixed systems			
	High production level	Medium production level	Low production level	All
Milk production level of the farm				
Production level of the mother, kg/year per head	300	200	100	-
Age at lamb slaughter, days <sup>1</sup>	30	30	30	30
Weight at slaughter, kg of LW <sup>1</sup>	10	10	10	10
Carcass weight <sup>1</sup>	6.5	6.5	6.5	6.5
				Milk replacer
Lamb feeding	Dam milk	Dam milk	Dam milk	
Feed conversion ratio, kg milk/kg LW <sup>1</sup>	5.5	5.5	5.5	5.5
Total milk consumption from birth to slaughter, kg	35.2	35.2	35.2	35.2
Emissions from milk production, kg of CO <sub>2eq</sub> /kg of milk	2.08 <sup>2</sup>	3.34 <sup>2</sup>	4.60 <sup>2</sup>	9.1 <sup>3</sup>
Enteric emissions, kg of CO <sub>2eq</sub> /kg of milk <sup>4</sup>	3.19	3.19	3.19	3.19
Manure emissions, kg of CO <sub>2eq</sub> /kg of milk <sup>4</sup>	0.270	0.270	0.270	0.270
Other emissions, kg of CO <sub>2eq</sub> /kg of milk	negligible	negligible	negligible	negligible
CF, kg of CO <sub>2</sub> per kg of live weight	7.7	12.1	16.5	32.5
CF, kg of CO <sub>2</sub> per kg of carcass weight	11.8	18.6	25.4	49.9
% from methane	4.2%	2.6%	1.9%	1.0%

<sup>1</sup> [34]; <sup>2</sup> Carbon footprint estimates from preliminary results of the EU Life15 project Forage4CLimate [32]; <sup>3</sup> Carbon footprint of milk replacer: considering milk replacer based on 60% of skimmed milk powder. Emissions were based values reported by Ecoinvent, 2018 using economic allocation methods and O'Brien et al. (2014) [35]; <sup>4</sup>Based on IPCC, 2006 [36]

#### 4. Innovative procedures able to preserve the traditional quality of lamb meat

The chemical composition of suckling lamb meat, which is rich in proteins, lipids and water, makes it a favourable substrate for the growth of microorganisms. Moreover, the lipid fraction, characterised by a sensible content of PUFA, also makes it very susceptible to oxidation. To succeed in international markets, the meat needs to be properly preserved as anoxic vacuum-packaged product and stored either chilled or frozen. Coombs et al. (2017) [37] in a recent review, reported the combination of conventional chilled (-1.5 to 5°C)-then-frozen is a valuable procedure for lamb and red meat storage for export purposes. Rapid chilling (at -40°C for about 2 h) followed by frozen storage is a promising innovation in meat storage. Rapid chilling is useful to reduce the meat temperature as soon as possible in order to prevent ice crystal formation before frozen storage, which then inhibits growth of microorganisms and avoids lipid peroxidation. We conducted a study to evaluate the rapid chilled-then-frozen procedure on lipid peroxidation of vacuum packaged suckling lamb meat (loins) when stored at two freezing temperatures. Loins from suckling lambs of the Sarda breed, covered by the Protected Geographical Indication (PGI) *Agnello di Sardegna*, were firstly vacuum packaged, then rapidly chilled and finally

assigned to two freezing temperatures ( $-12$  and  $-18^{\circ}\text{C}$ ) for 16 weeks. Results of the lipid oxidation, expressed as mg malondialdehyde (MDA)/kg, analysed in lamb muscle during frozen storage are reported in Figure 2. These results evidenced that the MDA levels did not differ between the two frozen storage temperatures, although they increased significantly from day 0 to 4 weeks and then remained stable until 16 weeks of storage. MAD levels were significantly increased from initial levels after 16 weeks of frozen storage, but remained constant until the end of the study. Final MAD levels, though, were much lower than MAD levels reported in lamb meat exposed to the conventional chilled-then-frozen procedure. The MDA level in our lamb loins was markedly below 2 mg MDA/kg, which is the threshold indicating little formation of secondary oxidation products responsible for the characteristic rancid smell of oxidised fats in foods [38]. This result strongly evidenced that this procedure should be a promising technique to preserve sensorial and nutritional properties of this meat.



**Figure 2.** Evolution of MDA values in muscle of lambs during storage at  $-12^{\circ}\text{C}$  and  $-18^{\circ}\text{C}$

## 5. Conclusions

Meat from suckling lambs fed maternal milk from their pasture-reared dams has very interesting and distinctive qualitative traits. The amount of PUFA $\omega$ -3 ingested by lactating ewes grazing on green pasture is able to increase the presence of beneficial FAs in their milk. This advantage improves the nutritional value of the meat derived from their suckling lambs compared to the meat of other lambs raised on milk replacers. Nowadays, scientific data on suckling lamb meat's composition and carbon footprint supports the allegation that this food is a functional and environmentally sustainable food. Moreover, innovative chill/freeze technologies are available to preserve the unusual nutritional properties of this food and allow its increased release to international markets and consumption.

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# Traditional and regional meat products in Poland

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**Abstract.** Nowadays, consumers in Europe are increasingly paying attention to the quality, health benefits and origin of meat products. Thanks to this, traditional and regional meat products, which in addition to being of high quality and part of regional/country traditions, are of great interest. European consumers are ever more willing to buy meat products produced using traditional methods from local raw materials, which – contrary to mass production – guarantees the highest quality.

## 1. Introduction

In the European Union countries, a retreat from food produced on an industrial scale has increasingly been noticed. Nowadays, people are much more aware of chemicals added to food and try to avoid them, switching to traditional and regional products. However, this is still a small segment of the food market [1]. Meat is an expensive but controversial commodity [18]. The policy of traditional and regional food quality in the European Union (EU) is implemented, among other factors, by distinguishing and certifying those foods that confirm the high quality of agri-food products originating from specific regions, as well as those characterized by traditional production methods [2]. This emphasizes the specific quality of regional and traditional food, increases consumer confidence in such foods and protects products from dishonest appropriation of the name, i.e., counterfeiting or food fraud. The production of traditional and regional foods is one of the most important factors affecting the development of rural areas [3]. It creates new livelihoods in the countryside, increases farmers' incomes, especially in less-favored areas, and prevents depopulation of these areas [4].

## 2. Traditional and regional products in Poland

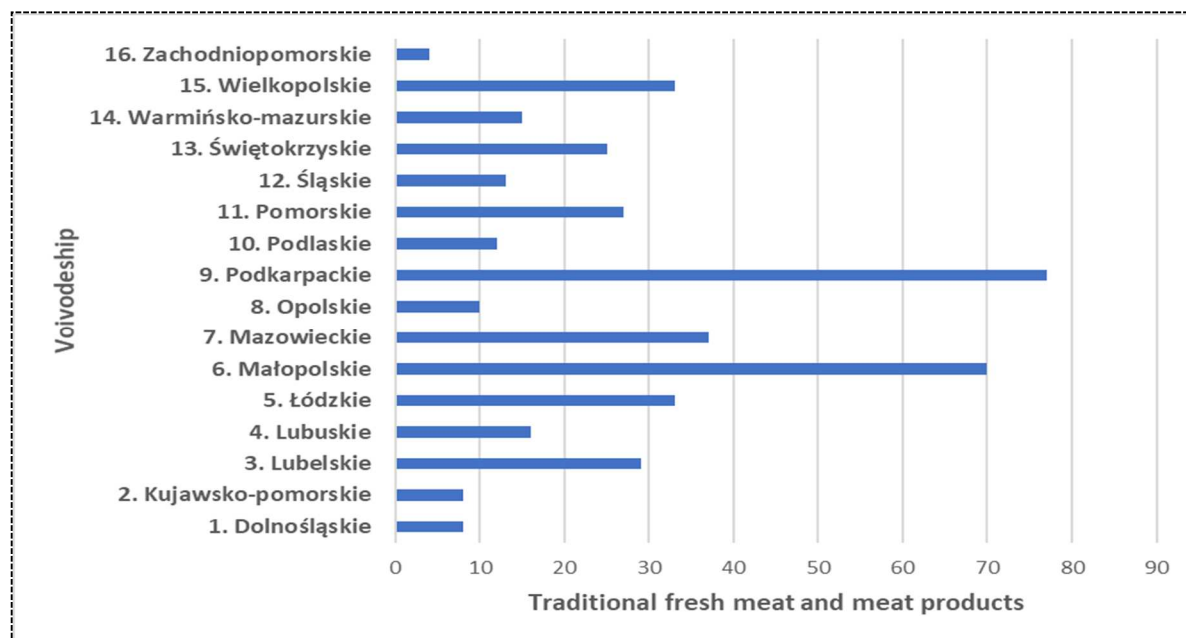
In order for a Polish product to be labeled traditional, it must be entered on the National List of Traditional Products. The National List of Traditional Products includes products that are characterized by traditional, time-honored production methods (recipes passed down from generation to generation), with quality or unique features and properties resulting from the use of traditional production methods. These are considered production methods used for at least 25 years as part of the heritage of the cultural region in which they are produced and as part of the identity of the local community [5, 12]. These traditional products are also the result of the influence of natural factors related to the area, such as climate, vegetation, terrain, and soil quality. Traditional products served under the umbrella of national or regional cuisines are a very important element of tourist promotion in both Poland and Europe [6]. Poland, as a member of the EU must meet the requirements imposed by EU law [10]. Actually, the system of promotion and protecting traditional and regional products in the EU is based on regulation of the European Parliament and of the Council (EU) No. 1151/2012 of November 21, 2012 *on quality systems for agricultural products of foodstuffs* and Supplementary Regulation of the Commission (EU) No 664/2014 of 18 October 2013 supplementary Regulation (EU) No 1151/2012 of the European Parliament and of the Council as regards the establishment of European Union symbols on protected designations of origin, protected geographical indications and



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guaranteed traditional specialties and with reference to some rules concerning the origin of feed and raw materials, some procedural provisions, and additional transitional provisions [6, 8]. The National List of Traditional Products (data from May 2019), currently shows that 417 meat products labeled *traditional* are registered in Poland (Figure 1) [9, 11].



**Figure 1.** Registered traditional fresh meat and meat products depending on their province in Poland [9]

### 2.1. *Kielbasa myśliwska* (hunting sausage) – Traditional Speciality Guaranteed

For the production of *kielbasa myśliwska* (hunting sausage), meat from pigs with a body weight of up to 120 kg is used, characterized by a higher intramuscular fat content (above 3%) than typical fatter pigs with weights of 90-100 kg (intramuscular fat content 2-3%). This more fatty type of meat is very useful for the production of traditional Polish sausages with their desired taste qualities. Slaughter should be based on traditionally maintained Polish breeds of pigs: *Puławska*, *Złotnicka*, *Wielka polska biała* or *Polska biała zwistoucha*. Mixed-breeds of these pigs can also be used. The minimum blood content of these native breeds should be 50%. Fattening is completed using traditional mixtures of cereal and other components [15]. Feed mixtures (doses) are composed of energy components including cereal meal – wheat, barley, rye, oat, wheat-rye, corn – and protein components – lupin, fava bean, pea meal, soy and rapeseed meal, rape cake, fodder yeast, dried green fodder. Feed can be supplemented with vitamins and minerals. The raw material for the production of *kielbasa myśliwska* is pork or 50% pork and 50% beef. The unique taste and smell distinguish *kielbasa myśliwska* from other types of sausages. This is the result of using carefully selected spices used (juniper, natural pepper, and fresh garlic), and sugar, curing mixture and a creamy mixture consisting of vinegar, water and rapeseed or sunflower oil. Juniper (*Juniperus*) is a traditional spice that occurs frequently in forests, which enhances the taste and smell of this sausage and significantly influences its specificity [14]. *Kielbasa myśliwska* is warm-smoked traditionally and heat-treated to achieve an internal temperature of at least 70°C. The smoking and heat-treating processes produce a characteristic color and unique taste, and the internal temperature of 70°C gives a uniform color and inactivates microorganisms present in the stuffing. The sausages are cooled to <10°C followed by drying at 14-18°C at 70% to 80% relative humidity for 5-7 days until the desired characteristics are obtained.

Strengthening and deepening of the external color then occurs through dehydration to obtain the desired shelf-life [16].

### 2.2. *Kabanos* – Traditional Speciality Guaranteed

The history of *kabanos* on Polish soil dates back to the 1920s and 1930s. *Kabanos* was made in small plants with local coverage under one name, but encompassing different regional varieties. The advantages of *kabanos* are the original taste and extended shelf life, which is ensured by smoking and drying processes. *Kabanos* is a thin, dried, smoked pork sausage in sheep intestine. The surface color is dark red with a shade of cherry. Dark red pieces of meat and light-cream cuts are visible on the cross-section fat. *Kabanos* has a distinctly perceptible taste of heat-treated, corned pork, and a light aftertaste of caraway, pepper and smoke. An important component of *kabanos* affecting its specificity is the pork from specially fattened pigs of up to 120 kg (as in the case of *kielbasa myśliwska*) and characterized by a higher intramuscular fat content [19]. *Kabanos* is produced from pork meat with an intramuscular fat content above 3%, which ensures appropriate taste and technological properties of meat [16]. The purpose of proper fattening is to obtain raw material (meat) characterized by an increased intramuscular fat content. For *kabanos* production, meat from traditional Polish pig breeds is used: *Puławska*, *Złotnicka*, *Wielka polska biała* or *Polska biała zwistoucha*. Apart from these pure-bred pigs, mixed breeds can also be used, but only those descended from the aforementioned breeds. This meat/fat raw material and compliance with the traditional production methods, with particular emphasis on the stages of grinding, curing and smoking, provides *kabanos* with exceptional crispiness (snap) and juiciness. Animals intended for this type of product are fed with natural feeds. The characteristic feature of *kabanos* is clearly heard when it is broken – a snapping sound is produced. This is the effect of meat fragility and properly conducted processes of drying and smoking. Smoking and heat-treating produce the characteristic color of the skin, the appropriate taste qualities, and heating to an internal temperature of 70°C destroys any pathogens present in the stuffing. The unique taste and smell of *kabanos* is the result of selected spices used (natural pepper, nutmeg, and cumin) and well-conducted drying, smoking and cooling processes [17].

### 2.3. *Kaszanka Nadwieprzanka* - Traditional Speciality Guaranteed

*Kaszanka Nadwieprzanka* is a well-known product in Poland. It is a blood sausage with buckwheat groats, scalded, and which includes masks (pork head rinds) and meat from pig heads, offal raw materials, pork skin, pork fat and blood [13]. *Kaszanka Nadwieprzanka* has a grainy cross-section with visible lighter groats and fat points, while the remaining mass is gray-brown or brown. The consistency is fragile, but the slices should not disintegrate, and the taste and smell are characteristic of offal sausage brewed with the addition of blood and buckwheat [20]. *Kaszanka* is moderately salty with perceptible aromatic spices. Raw materials used for the production of this kind of product are specially selected by contract with individual farmers from the Baranów commune; the meat and fat come exclusively from young pigs up to six months of age and weighing less than 100 kg. Water used for the production of this black pudding is naturally pure and derived from quaternary deposits. *Kaszanka Nadwieprzanka* has been produced since 1968 on the basis of the recipe developed by the management and crew of the *Masarnia Commune Samopomoc Chłopska*. The name of *Kaszanka Nadwieprzanka* dates from 1971, and came from the local jargon, referring to the restaurant *Nadwieprzanka* in Baranów where this blood sausage was served as a main snack. *Kaszanka* has a unique taste, a shelf-life of 7 days from the date of production, and is greatly appreciated by locals and foreigners alike [13].

## 3. Conclusions

The registration of meat products as Traditional Speciality Guaranteed is an excellent means to support regional promotion. Thanks to this registration system, meat products characteristic for particular regions have become showpieces, the efforts of the producers are rewarded and consumers have an opportunity to try authentic local cuisine. Traditional and regional food is a basic element

contributing to promotion of Polish cultural heritage. Promoting the cuisine and regional products is an obvious need in increasing the number of foreign tourists and in developing agri-tourism and tourism country-wide.

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# Sensory evaluation of meat and meat products: fundamentals and applications

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**Abstract.** Sensory analysis is widely applied to both fresh and processed meat. Herein, the frequently used sensory evaluation techniques are briefly characterised; some critical aspects, such as assessor selection, attribute generation, and context effects, are detailed. Without aiming to be exhaustive, some examples of previous research are show-cased to demonstrate what types of research questions can be answered by utilising sensory evaluation.

## 1. Sensory evaluation techniques

It is fundamental to think about the scope of any sensory test, as there exist various approaches serving to answer distinct questions. Typically, sensory evaluation techniques are categorised in three fields:

- Discrimination testing (a.k.a. difference testing);
- Descriptive analysis;
- Consumer acceptance and preference testing.

### 1.1. Discrimination testing

Discrimination testing can help to achieve confidence as to whether or not sensory differences are perceivable to consumers or trained assessors. This type of testing is applied if little or no sensory difference between a set of samples is suspected. Difference tests can be conducted either generically or with prior specification of the attribute of interest. That is, one is interested in whether there is any perceivable difference or whether products differ, for example, in sweetness. Various standardised discrimination tests that differ in their complexity and their sensitivity/power are available such as the triangle or tetrad test (unspecific), 2-AFC and 3-AFC test (attribute specific).

### 1.2. Descriptive sensory analysis

Descriptive sensory analysis typically is used when one is interested both in the direction and in the magnitude of differences. That is, the aim is to identify the relevant sensory attributes of a given product or in which attributes the various products differ. Therefore, a trained panel consisting of selected assessors (typically 8-12 assessors) is used to develop a sensory lexicon for further quantitative assessment of products using that lexicon and (typically) line scales indicating the intensity of a given attribute.



### 1.3. Consumer acceptance testing

Consumer acceptance testing aims at identifying likes and dislikes for a given set of samples. Therefore, a large consumer sample (typically  $n > 60$  consumers) is used and asked to indicate liking/disliking using hedonic scales. The most common hedonic scale is a 9 point anchored scale, i.e. with verbal labels for each category. If only two samples are studied, pairwise comparisons are applicable to illuminate consumer preferences; this is also possible whenever several samples are to be compared to a constant reference, as was done for boar tainted meat vs. castrate meat <sup>1</sup>.

Frequently, it is important to understand which sensory attributes affect consumer acceptance. Therefore, to identify so called “drivers of liking”, liking data obtained through consumer testing is modelled via PLS-regressions or other multivariate approaches using sensory descriptive (profiling) data obtained from trained assessors.

## 2. Applications

Sensory analysis is widely applied to both fresh and processed meat. Without being exhaustive, some examples of sensory evaluation applied to meat are given as follows:

A recent study investigated the effect of differing marbling levels in beef strip loin steaks (all considered tender) on sensory evaluation by trained panellists and consumers' acceptability. In the results, beef flavour scores increased with increasing fat level (2 to 26% fat), which was also the primary driver of acceptability <sup>2</sup>. Hence, this study confirms the importance of flavour to beef palatability, regardless of tenderness.

Descriptive sensory analysis was used to investigate warmed-over flavour (WOF) development in cooked, chill-stored and reheated pork patties <sup>3</sup>. Multivariate data analysis (Analysis of Variance–Partial Least Squares Regression, ANOVA–PLSR) was used to determine the association between the design variables (storage duration before reheating and pre-slaughter stress level of the pigs), and sensory and chemical data. As per the study, WOF was attributable to the development of lipid oxidation-derived off-flavour and odour notes, e.g. rancid-like flavour and linseed oil-like odour, in concurrence with a decrease in perceived meatiness, i.e., cooked pork meat-like flavour. The sensory variation related to pre-slaughter stress appeared to be distinct from WOF variation.

A very recent study researched the use of alternative proteins for livestock feeding to elucidate consequences for resulting meat quality including sensory traits <sup>4</sup>. Pork quality characteristics related to the dietary substitution of soybean meal with the micro-alga *Spirulina* (*Arthrospira platensis*) or black soldier fly (*Hermetia illucens*) partly-defatted larval meal were investigated, which included sensory profiling by trained assessors. The results showed that meat quality is not compromised by including these alternative protein sources in pig diets. Other fields of research involving sensory evaluation of fresh meat involve effects of breed, diet, husbandry systems, pre- and post-slaughter handling, packaging and ageing – to name a few.

Sensory evaluation techniques are also frequently applied to meat products. For example, quantitative descriptive analysis (= sensory profiling) was used to characterise traditional sausages produced in the Massif central region in France <sup>5</sup>. Sensory data were correlated with information regarding the production obtained from the producers *via* a questionnaire. Thus the relationship of recipe/manufacturing and resulting sensory properties of the final products was established; discriminant analysis revealed it was possible to classify the sausages correctly into specific manufacturing practice groups based on the sensory data. Hence, this approach can be used for quality labelling such as protected designation of origin/ protected geographical indication (PGI/PDO).

Other applications include the reduction of salt, fat and sugar in meat products, modification of the fatty acid composition by adding plant oils, the use of new ingredients for meat processing (healthy meat products such as hydrolysed by-products or fibres with potential health benefits <sup>6</sup>) and the use of boar tainted meat <sup>7–9</sup>.

### 3. Assessor selection and performance

#### 3.1. Descriptive analyses

For the objective assessment of fresh meat, guidelines for selection and training of assessors are publicly available. For example, exercises are outlined to screen assessors' ability to detect difference in juiciness, tenderness or flavour of meat <sup>10</sup>.

For the evaluation of boar taint, it is critical to select appropriate assessors for sensory evaluations. It is well known that people differ tremendously in their olfactory acuity for androstenone, a key compound of boar taint, which in turn affects their perception of boar taint in meat; this also holds for consumers and their hedonic judgment of boar meat <sup>11,12</sup>. Standardised smell tests can be applied to characterise both trained assessors and consumers <sup>1,13</sup>. Therefore, it is suggested assessors are chosen according to their olfactory acuity for the key boar taint compounds, androstenone and skatole.

For research, their performance should be described as one would describe, e.g., the limit of detection of a gas chromatography protocol used. For critical applications, such as the detection of boar taint at slaughter (for sorting carcasses), performance criteria such as being under the receiver-operating characteristic (ROC) curve or intra-class correlations of a panel or evaluation method should be indicated <sup>14</sup>.

#### 3.2. Hedonic analyses

For the hedonic assessment of meat and meat products, consumer panellists must be recruited. Depending on the aim of the project, demographic and socio-economic criteria (e.g. age, gender, household size, income, area of residence), usage (e.g., heavy vs. light users) and attitudes are considered. Typically, one aims to address consumers of the products to be tested. Since consumers' hedonic ratings vary to a greater extent than trained panellists' ratings, large sample sizes are needed to show significance of product effects.

It is important to keep in mind that panellists trained for descriptive sensory analysis must under no circumstances evaluate products hedonically – this is one central paradigm of sensory analysis. However, this mistake is frequently to be found in practice and in academia.

### 4. Sample preparation and presentation

Depending on the aim of the project, a protocol for the sensory study needs to be chosen. This includes aspects such as product sampling, sample preparation and sample presentation. For fresh meat evaluation, frequent cooking procedures include braising, broiling, electric charbroiling, roasting, grilling, outdoor grilling and sous-vide cooking. Detailed descriptions are, for instance, provided in the American Meat Science Association (AMSA) guidelines. For example, sous-vide cooking is used to identify small flavour differences by avoiding additional aroma development from Maillard reaction. The amount of sample presented to panellists for one test can differ substantially (e.g. from presenting a whole steak to small cubes of meat). The size should be big enough so that panellists can evaluate the given sample. Additionally, sensory fatigue and satiety need to be taken into account. The presentation order should, whenever possible, be balanced or at least randomised to avoid first position effects and carry-over effects. Especially for consumer studies, a systematic bias of scores for the first sample is frequently observed.

### 5. Sensory descriptors

The AMSA provides guidelines for cooking, sensory evaluation and instrumental tenderness measurements of meats including a generic scheme for descriptive meat analysis <sup>10</sup>. Whenever possible, such a generic scheme should be replaced with a species-specific flavour lexicon, whereby each flavour attribute can be referenced and scaled <sup>15</sup>. If this is not practical or not necessary based on the experiment objectives, one or two flavour notes, such as beef flavour identity (=the amount of beef flavour), could be used instead. The development and application of a set of sensory descriptors was demonstrated earlier to derive a sensory landscape of 15 meat species <sup>16</sup>, so this could well serve as a

basis. If texture of the meat is of special interest, a detailed vocabulary can be found elsewhere <sup>17</sup>. For the case of boar taint, specific descriptors such as sweaty, urine, manure and mothball have been identified and are being frequently used <sup>9,18</sup>.

## 6. Consumer ballots

Typically, consumer ballots comprise first order questions (“How do you like/dislike this product overall?”) and second order questions (“How do you like the tenderness of this steak?”) for further diagnostics. In addition, just about right (JAR) scales can be applied to identify how consumers perceive certain attributes and how that affects their hedonic evaluation. To do so, usually a 5 point category scale is used, and consumers indicate whether a specific attribute’s intensity is too low, too high or just right. Penalty analysis is used to assess the impact of a product being not JAR by calculating the overall liking drop. For further product diagnostics, check-all-that-applies (CATA) has been increasingly applied in research to speed-up product optimisation processes. Therein, consumers indicate their overall liking and record their perception of various attributes. Penalty analysis on CATA items proved to be a simple and useful approach to identify drivers of liking and directions for improving the products <sup>19</sup>.

## 7. Non-inferiority testing

When reformulating products, replacing unwanted ingredients or changing processing technology, it is often rather desirable that consumer liking stays the same as before. For such research questions, non-inferiority testing <sup>20</sup> is suggested rather than looking at significant differences. Non-inferiority testing is suitable whenever a formulation is to be identified that is as similarly acceptable as the current standard. For example, a consumer study identified the maximum allowable proportion of tainted boar meat for Frankfurter type sausages without significantly impairing consumer acceptance <sup>7</sup>.

## 8. Context

Frequently, the effect of labels (e.g., organic, country of origin, production system) on sensory consumer acceptance is studied. This is because meat and meat products typically are sold with such labels on the pack. Strikingly, such labels often improved the acceptance scores, even though the underlying meat was not scored significantly differently under blind conditions. In the case of boar taint, it was demonstrated that labelling the meat as originating from “young boars” did not decrease consumer acceptance <sup>14</sup>. On the contrary it was demonstrated repeatedly that labelling products as “organic” or “free range” increases consumer acceptability scores <sup>21,22</sup>.

Another aspect is consumption context. Occasionally, the product of interest is presented in a meal context to evaluate its perception in a more realistic situation than presenting the product alone. A comparative study on consumer acceptance of pork with differing levels of boar taint found, however, no difference regardless of whether the meat was presented with or without side dishes <sup>23</sup>. The study also showed that sensory defects detected by trained panellists may not be noticed by untrained, and thus usually less sensitive, consumers.

Also, the testing surroundings itself provides context that can affect consumer ratings. Emerging immersive technologies (e.g. virtual reality, VR glasses or screens) are being researched as to whether they could help to increase the external validity of sensory consumer testing by providing the consumption context more realistically than classical sensory laboratory testing.

## 9. Conclusion

If applied correctly, sensory science is a powerful tool, as it provides measures no other instrument (to date) can provide: a detailed description of how a food item is perceived by the human senses and/or how much this item is liked. It is, therefore, fundamental to think about the scope of any sensory test and to consider good sensory practice.

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## Pros and cons of using a computer vision system for color evaluation of meat and meat products

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**Abstract.** The ability of a computer vision system to evaluate the color of meat and meat products was investigated by a comparison study with color measurements from a traditional colorimeter. Pros and cons of using a computer vision system for color evaluation of meat and meat products were evaluated. Statistical analysis revealed significant differences between the instrumental values in all three dimensions ( $L^*$ ,  $a^*$ ,  $b^*$ ) between the computer vision system and the colorimeter. The computer vision system-generated colors were perceived as being more similar to the sample of the meat products visualized on the monitor, compared to colorimeter-generated colors in all (100%) individual trials performed. The use of the computer vision system is, therefore, considered a superior and less expensive alternative to the traditional method for measuring color of meat and meat products. The disadvantages of the computer vision system are its size, which makes it stationary, and the lack of official manufacturers that can provide ready-to-use systems. This type of computerized system still demands experts for its assembly and utilization.

### 1. Introduction

Color is primarily a personal experience. Without color, visual and emotional experiences we have while looking at the world around us, including our food, is imperfect. For most foods, visual appearance is the first thing to have a sensory impact on us. Color influences meat-purchasing decisions as well. For consumers, a major indicator of meat's freshness and wholesomeness is discoloration, making color a major meat quality factor [4]. This information is apparent to meat producers, retailers and researchers in meat science and technology. The importance of color is also reflected in the fact that improving color stability of meat and meat products will influence their shelf life by increasing the time that meat is still visually acceptable to consumers at retail [5]. To ensure food conformity to consumer expectations, it is critical for the food processing industry to develop effective color inspection systems to measure the color information of food products. Traditionally, instrumental meat color is assessed with a colorimeter [6]. However, all colorimeters have the



disadvantage that the surface to be measured must be uniform and rather small ( $\sim 2\text{-}5\text{ cm}^2$ ) [7], which influences bias in measurements. Another problem is that optically non-homogeneous media such as poultry meat, refract, reflect, diffuse and absorb the light beam emitted by the colorimeter [8], causing deviations in all color dimensions evaluated. Therefore, to measure food color rapidly and non-invasively, new, objective and consistent methods are required. Among numerous new sensing technologies for the assessment of agricultural and food products, the computer vision system (CVS) is a novel technology for food color evaluation. The aim of this review is to present the application of a CVS for instrumental color evaluation of poultry and game meat and meat products with various physical properties and its advantages over the traditional color measuring method.

## 2. Materials and methods

### 2.1 Samples of meat and meat products

The research was carried out on *m. pectoralis major* samples of three animals for each of the four poultry species (chicken, turkey, duck and goose) and five game meat species (quail, wild boar, rabbit, deer and pheasant). We selected the samples in a retail setting. Before color analysis, freshly cut meat samples, about 3 cm thick, were individually placed on white polystyrene foam trays with a consistent color and overwrapped with a transparent PVC film permeable to oxygen. Then they were placed in a bench refrigerator at 4 °C for 30 min to obtain myoglobin oxygenation. The PVC film was removed before color measurement.

In addition, a group of meat products including the fresh processed, raw cured, cooked cured, raw cooked, and precooked-cooked categories and raw (dry) fermented sausages were investigated. Based on the treatment of raw materials and the individual processing steps and taking into account the processing technologies used, it is possible to classify processed meat products into these six broad groups of processed meat products [9]. In our research, within each product category, there were at least two and a maximum of four representative samples, so altogether, 18 different meat products were investigated.

### 2.2 Minolta CR-400 colorimeter

A Minolta CR-400 colorimeter with 8 mm aperture, 2° observer, illuminant D65 and pulsed xenon lamp was used as a default light source. A glass cover was applied over the aperture port while measurements were taken. The device was calibrated before each analysis with a standard white tile.

### 2.3 Computer vision system (CVS)

A Sony Alpha DSLR-A200 digital camera (10.2 Megapixel CCD sensor) was used. The camera was located vertically at a 30 cm distance from the sample. The camera setting was the following: shutter speed 1/6 s, manual operation mode, aperture Av F/11.0, ISO velocity 100, flash off, focal distance 30 mm, lens: DT-S18-70 mm f 3.5-5.6. Four Philips fluorescent lamps (Master Graphica TLD 965) with a color temperature of 6500 K were used for lighting. Each lamp was equipped with a designated light diffuser. In order to achieve uniform light intensity on the meat samples, the lamps (60 cm length) were located at a 45° angle and 50 cm above the samples. Both the lamps and the camera were fixed inside a cubic ( $a = 80\text{ cm}$ ) wooden box with a removable top [2]. The box had an opening to the side for sample entry and another on the top for visual inspection before and after the measurements. The internal walls of the box were coated with black opaque photographic cloth to diminish background light.

After the camera and the monitor were calibrated, as explained in the investigation of Tomasevic *et al.* [2], the Adobe Photoshop CC (64 bit) software was used for image analysis. The colorimetric characteristics from RGB images were acquired using RAW photographs. They were measured on the digital image of the sample, using the Photoshop Average Color Sampler Tool (image area analyzed: 31 x 31 pixels).

### 2.4 Color changes

Total color difference ( $\Delta E$ ) was determined by using the standard equation:

$$\Delta E = \sqrt{(a_C^* - a_M^*)^2 + (b_C - b_M^*)^2 + (L_C^* - L_M^*)^2} \quad (1)$$

Values for  $a_C$ ,  $b_C$ ,  $L_C$  were obtained from the meat products using CVS, and for  $a_M$ ,  $b_M$ ,  $L_M$  using the Minolta colorimeter.

The degree of difference of hue as the quantitative attribute of colorfulness chroma ( $C^*ab$ ) was calculated according to Fernández-Vázquez *et al.* [10]:

$$C^* = \sqrt{a^2 + b^2} \quad (2)$$

The difference in Chroma  $\Delta C$  and lightness  $\Delta L$  values were calculated using standard formulas:

$$\Delta C = C_C^* - C_M^* \quad (3a)$$

$$\Delta L = L_C^* - L_M^* \quad (3b)$$

Hue difference  $\Delta H$  was calculated according to Mokrzycki and Tatol [13]:

$$\Delta H = \sqrt{\Delta E^2 - \Delta L^2 + \Delta C^2} \quad (4)$$

## 2.5 Similarity tests

The tests used were adopted from the investigation of [8] with slight modifications. For all the tests performed, 14 panelists were individually seated at a distance of approximately 60 cm from the calibrated monitor, equipped with a shade that reduces glare (Compushade Universal Monitor Hood, DulCO, USA). Similarity tests were: test A – respondents compared photographs and real meat samples; test B – matching test: which chip is more close to the photo of the meat; test C – degree of difference of the color chips.

For test A, panelists were asked to compare the color of a digital image displayed on the monitor and a meat sample presented on polystyrene trays. They had up to 30s to rate the similarity by answering “yes” or “no”. If yes, the panelists had the opportunity to indicate the level of similarity according to a five-point Likert scale from 1 “very low”, 2 “low”, 3 “moderate”, 4 “high” to 5 “very high”.

Test B involved displaying colors generated by Adobe Photoshop CC (2015) using the  $L^*$ ,  $a^*$  and  $b^*$  values obtained from both the CVS and Colorimeter (Minolta) data together on the monitor and panelists were asked to evaluate which of the two generated color chips was more similar to the sample of the product visualized on the monitor.

During test C, the panelists were asked to evaluate the level of difference between the two color chips (colorimeter and CVS) displayed on the monitor and rank the difference according to a five-point Likert scale from 1 “very low”, 2 “low”, 3 “moderate”, 4 “high” to 5 “very high”.

## 3. Results and discussion

### 3.1 Poultry meat

Consumers often select chicken meat based on its color, as it has significant influence on how they perceive quality characteristics of chicken meat products [11]. Because for the meat producers improving quality and customer satisfaction is a major objective [12], they also pay special attention to its color. The  $L^*$ ,  $a^*$ ,  $b^*$ , chroma and hue angle values of poultry meat, measured with CVS and colorimeter in our experiment, were significantly different [1]. The magnitude of color difference between the two pieces of equipment used is best represented by the total color difference value ( $\Delta E$ ). A clear threshold for human ability to detect meat-color difference has not been established, but a possible value could be around 2-6 [14].  $\Delta E$  in the range from 2 to 10 indicate the difference in color is perceptible at a glance and when  $\Delta E$  is larger than 10, we can conclude that colors are more



**Figure 1.** Color of poultry meat as measured by the two methods [1]



**Figure 2.** Color of game meat as measured by the two methods [3]

opposite then similar [15]. Therefore, with the  $\Delta E=18.5$  for chicken meat and  $\Delta E= 22.04$  for turkey meat observed in this study, we can conclude that the two systems measured the color of chicken meat significantly differently, and even contrasting [1]. Positive  $\Delta L$  values indicate that the color measured with the CVS was lighter than the color obtained with colorimeter (Figure 1). However, the total color differences ( $\Delta E$ ) between the two methods were, for duck and goose, half the values calculated for chicken and turkey. Yet, with  $\Delta E$  values above 10 [1], these differences in color should be perceptible at a glance or considered more opposite then similar. Negative  $\Delta L$  values for duck and goose breasts indicate that the color measured with the CVS was darker than the color obtained with the colorimeter (Figure 1).

### 3.2 Game meat

Color of game meat plays a crucial role for many of its European consumers [16]. Game meat is a darker

red in appearance than meat from domestic animals, and is characterized by low  $L^*$  values below 40, high  $a^*$  values and low  $b^*$  values which are indicative of the dark red color [17]. However, the  $L^*$ ,  $a^*$  and chroma values measured with CVS and colorimeter in our study were significantly different [3]. Negative  $\Delta L$  values for wild boar and deer meat indicate that the color measured with the CVS was darker than the color obtained with the colorimeter. All the  $a^*$  values were higher when measured with the CVS compared to the colorimeter, meaning that the color obtained with the CVS was more “red” (or less “green”) (Figure 2). No statistically significant differences between the two applied methods were observed for  $b^*$  and hue angle values. It is evident that differences in meat color and color

stability between species can largely be attributed to differences in their muscle activity, which influences the muscle fiber type, myoglobin concentration and intramuscular fat content of the meat, which in turn influence the muscle color. Therefore, not all game meat is darker in color than meat from domestic animals [18].

The instrumental color values ( $L^*$ ,  $a^*$ ,  $b^*$ , chroma and hue angle) obtained with the CVS for lighter colored game meat samples (quail, pheasant and rabbit) were statistically different from the same values obtained with the colorimeter [3]. Positive  $\Delta L$  values indicate that the color measured with the CVS was lighter than the color obtained with the colorimeter. All the  $a^*$  values were much higher when measured with the CVS compared to the colorimeter, meaning that the color obtained with the CVS was more “red” (or less “green”) (Figure 2). The positive difference in chroma ( $\Delta C$ ) meant that the CVS-generated color of quail and rabbit had greater intensity (were more saturated) than colorimeter-generated colors [3]. The CVS-generated colors were in a clockwise direction from colorimeter-generated colors, representing a shift in the red direction (Figure 2), since all the hue angle values were significantly higher when measured with the colorimeter compared to the CVS. The  $\Delta E$  values ranged from 9.67 to 19.01, indicating that for lighter colored game meat samples, the two systems measured their color significantly differently [3] and in the case of rabbit meat, even contrastingly.

### 3.3 Meat products

When the color of uniformly-colored meat products was evaluated, the total color difference value ( $\Delta E$ ) ranged from 6.7 for saveloy sausage up to 26.0 for pork prosciutto. For the majority of meat products with homogenous surfaces,  $\Delta E$  was around 10 [2]. Positive  $\Delta L$  values for uniformly-colored meat products indicate that the color measured with the CVS was lighter than the color obtained with the colorimeter. All the  $a^*$  values were higher when measured with the CVS compared to the colorimeter, meaning the colors obtained with the CVS were more “red” (Figure 3), and with the exception of pork prosciutto and raw sausage, all the  $b^*$  measured with the colorimeter were significantly higher than the values obtained with the CVS [2], meaning the colors of uniformly-colored meat products acquired with the CVS were more “blue” (or less “yellow”) compared to colorimeter-acquired colors (Figure 3). The positive difference in chroma ( $\Delta C$ ) meant that the CVS colors of cooked ham, pork and beef prosciutto and raw sausage had greater intensity or were more saturated than colorimeter-generated colors [2]. The opposite was observed for the beef, chicken and liver pate, smoked-cooked pork, frankfurter and saveloy sausage. Our investigation is in concurrence



**Figure 3.** Color of uniformly colored meat products as measured by the two methods [2]



**Figure 4.** Color of bi and non-uniformly colored meat products as measured by the two methods [2]

with the conclusions of Valous *et al.* [19], who found CVS to be a tool that can objectively specify color of cooked-hams.

Bi-colored meat products, like mortadella, bacon, dry pork neck or pancetta, consisted of meat and fat segments that were larger than the Minolta aperture size (8 mm) used in our study, allowing the colorimeter to measure their color independently. The total color differences between the two methods for the meat segments ranged from 7.3 to 14.6 and for the fat parts ranged from 7.7 to 12.9 [2]. Meat segments were assessed as having darker and fat segments as having lighter colors when measured with the CVS compared to the colorimeter (Figure 4a). Non-uniformly colored meat product was any product that had meat and fat parts that were too small (less than 8 mm) for the colorimeter to independently assess their color. Therefore, when the color of beef and pork fermented sausage, and hamburger was measured, the  $L^*$ ,  $a^*$ ,  $b^*$  colorimeter-generated values for both meat and fat parts were the same. Because the CVS used 31 x 31 pixels for the average color sampler tool, it was capable of measuring the color of meat and fat parts independently in these non-uniformly colored meat products. This resulted with the highest total meat-parts color difference ( $\Delta E = 20.3$ ), measured for beef fermented sausage, and maximum total fat-parts color difference ( $\Delta E = 35.3$ ), measured for pork fermented sausage [2]. These extraordinarily high values for total color differences [20] indicated the colors assessed by the two methods were almost exact opposites [15]. The color of meat parts measured with the CVS were significantly darker, had greater intensity and were more saturated, compared to colorimeter-measured equivalents (Figure 4b). The opposite was observed for CVS-generated fat color. Due to the high variability and complex color distribution in non-uniformly colored meat products, the colorimeter was unable to accurately assess the color of the meat parts and the color of the fat parts. Instead, the colorimeter produced  $L^*$ ,  $a^*$ ,  $b^*$  values that were somewhere in between the values for these two tissue segments. Our investigation is in concurrence with the conclusions of [21], who concluded that CVS is a tool that can objectively evaluate color of fermented sausages.

**Table 1.** Similarity tests results

	Frequency of similarity (test A)	Level of similarity (test A)	CVS vs. Colorimeter (test B)	Level of difference (test C)
Beef pate	100%	$3.4 \pm 1.4^{a,b}$	CVS (100%)	$3.0 \pm 1.1^{a,b,c}$
Liver pate	100%	$3.6 \pm 1.1^{a,b}$	CVS (100%)	$2.4 \pm 1.1^{a,b,c}$
Chicken pate	92.9%	$3.5 \pm 1.0^{a,b}$	CVS (100%)	$2.1 \pm 1.0^{a,b,c}$
Beef fermented sausage	92.9%	$3.6 \pm 1.0^{a,b}$	CVS (100%)	$3.2 \pm 0.4^{a,b,c}$
Pork fermented sausage	100%	$4.0 \pm 0.8^{a,b}$	CVS (100%)	$2.3 \pm 0.5^{a,b,c}$
Frankfurter	100%	$4.0 \pm 1.1^{a,b}$	CVS (100%)	$1.7 \pm 0.5^{a,b}$
Saveloy sausage	100%	$3.8 \pm 0.9^{a,b}$	CVS (100%)	$1.2 \pm 0.5^a$
Mortadella	100%	$2.9 \pm 1.2^a$	CVS (100%)	$2.1 \pm 1.1^{a,b,c}$
Cooked ham	100%	$3.0 \pm 1.2^{a,b}$	CVS (100%)	$3.6 \pm 0.3^{b,c}$
Smoked cooked bacon	92.9%	$3.1 \pm 1.3^{a,b}$	CVS (100%)	$2.2 \pm 0.4^{a,b,c}$
Smoked cooked pork	100%	$3.5 \pm 1.0^{a,b}$	CVS (100%)	$2.8 \pm 1.2^{a,b,c}$
Pork prosciutto	100%	$4.1 \pm 0.8^{a,b}$	CVS (100%)	$4.2 \pm 1.0^c$
Beef prosciutto	100%	$3.6 \pm 0.9^{a,b}$	CVS (100%)	$3.1 \pm 1.8^{a,b,c}$
Dry pork neck	92.9%	$3.5 \pm 1.3^{a,b}$	CVS (100%)	$3.0 \pm 0.7^{a,b,c}$
Pancetta	92.9%	$2.8 \pm 1.5^a$	CVS (100%)	$2.7 \pm 1.5^{a,b,c}$
Pork hamburger	100%	$2.8 \pm 1.0^a$	CVS (100%)	$2.0 \pm 1.0^{a,b,c}$
Beef hamburger	100%	$3.4 \pm 1.3^{a,b}$	CVS (100%)	$2.7 \pm 1.0^{a,b,c}$
Raw sausage	100%	$4.4 \pm 0.8^b$	CVS (100%)	$3.2 \pm 1.5^{a,b,c}$
Chicken breast	100%	$1.7 \pm 0.8^a$	CVS (100%)	$3.8 \pm 1.4^a$

Duck breast	100%	$2.4 \pm 1.0^{a,b}$	CVS (100%)	$1.8 \pm 0.4^b$
Goose breast	100%	$3.1 \pm 0.8^b$	CVS (100%)	$1.4 \pm 0.5^a$
Turkey breast	100%	$2.9 \pm 1.03^b$	CVS (100%)	$4.7 \pm 0.7^b$
Quail	100%	$2.7 \pm 1.3^a$	CVS (100%)	$3.6 \pm 1.4^a$
Wild boar	100%	$3.4 \pm 1.3^b$	CVS (100%)	$1.9 \pm 0.9^{b,c}$
Rabbit	85.7%	$2.7 \pm 1.2^a$	CVS (100%)	$4.2 \pm 1.2^a$
Deer	100%	$4.1 \pm 0.8^b$	CVS (100%)	$1.0 \pm 0.0^c$
Pheasant	100%	$3.2 \pm 1.2^{a,b}$	CVS (100%)	$3.4 \pm 1.3^{a,b}$

Means in the same column with different small letters are significantly different ( $P < 0.05$ ); Five-point Likert scale ranks from 1 “very low”, 2 “low”, 3 “moderate”, 4 “high” to 5 “very high”

### 3.4 Similarity tests

The results of the first similarity test (test A) between the color of the actual sample of meat products and the CVS-generated color of the image displayed on the monitor showed that the panelists found the digital images similar to the actual samples ( $P < 0.001$ ). The frequency of similarity assessed by the panelists was 100% for all poultry meat and game meat samples (Table 1). This means that 14 out of 14 panelists found that the actual color of all samples was similar to the chip color generated by the CVS. The frequency of similarity for meat products was also very high and ranged from 92.9% for chicken pate, beef sausage, smoked bacon, dry pork neck and pancetta, to 100% for all the other meat product samples. For poultry meat samples, the level of similarity ranged from “low” to “moderate” and for game meat and meat products samples from “moderate” to “high”.

Test B showed the CVS-generated color chips were more similar to the samples of poultry meat, game meat and meat products visualized on the monitor than to colorimeter-generated color chips in all (100%) individual trials performed (Table 1).

Test C, regarding meat products, revealed that, as assessed by the panelists, the magnitude of differences between the color chips generated by the CVS and the colorimeter and displayed on the monitor ranged from 1.2 (“very low”) for saveloy sausage to 4.2 (“high”) for pork prosciutto. The highest level of difference between colors for poultry meat was observed in the case of turkey meat (4.7 – “very high”) and for game meat samples with rabbit (4.2 – “high”).

## 4. Conclusion

We presume that one parameter influencing the difference between the two methods employed to measure the color of meat and meat products could be the penetration depth of the illumination source. In our investigation, the light employed in both devices had the same color temperature (6500 K), but the light interaction with the samples was obviously device-dependent. For the same reasons as were reported in an earlier meat color study [8], we deem the colorimeter not suitable for the color analysis of meat products. The reason is the translucent and optically non-homogenous matrix of the meat products due to the presence of different ingredients scattered inside these foods. The colorimeter is placed on the sample surface and the light penetration through the meat product matrix is required to be higher than for CVS. This, therefore, causes multiple reflections and refractions where optical discontinuities are present, resulting in a diffusion of light (scattering) from the illumination source [22], making the colorimeter measurements unsuitably inaccurate.

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# From white coat and gumboots to virtual reality and digitalisation: where is veterinary medicine now?

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**Abstract.** The paper reviews the current role of information and communication technologies in veterinary medicine, management of animal health, animal production and food safety worldwide and discusses the shift from recognising the digital revolution as a novelty to accepting it as a norm. Due to the diversity in veterinary medicine, it is unlikely that information and communication technologies will fully substitute the need for field veterinarians in direct contact with animals, farms, diseases, food production and food products. However, information and communication technology has a growing role in our work and provides opportunities to exploit new technologies for professional and societal affirmation. Consumers, trade and regulations drive demands on veterinary medicine, reflected in our increased focus on prevention and early recognition of animal diseases and food safety issues through output-based and integrated monitoring systems and shared responsibility between public and private sectors. Simultaneously, information and communication technology has been incorporated within these demands and so now has roles in: veterinary clinical practice, herd health management, animal health databases, traceability of animals and their products, trade and veterinary certification, animal disease data analysis, tools for veterinary education and animal health diagnostics. The symbiosis of bio- and information technologies has opened a new era in health and food production, providing a novel chance for veterinarians to make a significant leap in their professional development, achievable only through strategic and active participation as leaders and collaborators.

## 1. Global convergence through informatization

During the 1970s, a decade when the first email was sent, SONY launched its Walkman and the digital camera was introduced, the German philosopher Martin Heidegger stated: “Everywhere we remain unfree and chained to technology, whether we passionately affirm or deny it.” The continuing convergence of telecommunications, computers and digitisation has left nations – big or small – with little option but to informatize so they can effectively exist and compete in the world’s economy [1]. Information and communication technologies (ICT) have now completely changed operations for example, in the banking and tourism sectors, evident if we just compare how we manage our personal banking and book travel or accommodation now and just a few decades ago. ICT has not just influenced business and economic aspects, but also societal aspects, with new tools for communication (social networks) and the increased availability of information in the contexts of democracy, globalisation, human rights and social responsibility issues.

As animal health, welfare and food safety professionals we can readily acknowledge consumers, trade and regulations drive the changes in our everyday work. Today, we are more specialised and versatile in the tasks we do, more output driven, more publicly responsible regarding the approaches and tools we use and more conditioned by scientific and cost-effective justification for our decisions. Our professionally specific challenges are already interlinked with the ICT framework of our modern



society and economy, and the question arises, are we open to recognise and (pro)actively react to the possible threats, opportunities and novel roles these technologies offer?

This paper reviews the role of ICT in current operations within veterinary medicine, management of animal health, animal production and food safety worldwide and discusses the shift from recognising the digital revolution as a novelty to accepting it as a norm.

### *1.1. Informatization and veterinary medicine, animal health and production and food safety*

It is easy, from today's perspective, to recognise the importance, developments and roles of veterinary medicine in the agricultural age or even the industrial age. The period when agriculture was the predominant factor shaping societies, governance, economies and life (agricultural age) ended by 1750 with the agricultural revolution during the 18<sup>th</sup> and early 19<sup>th</sup> centuries that introduced industrialised food production through the technological improvements brought about in the industrial age. During the industrial age, veterinary medicine made a huge leap forwards by rebranding itself from a vocation to a profession (marked by founding the first veterinary school in Lyon in 1762) and constantly expanding its subject matter (from only horses to cattle and other livestock, followed by companion animals, aquatic animals, insects as a source of food, wildlife, zoonotic agents, environmental health, integrated and interdisciplinary chains of food production and control, veterinary public health) [2]. From the 1950s, the information age (also called the digital age) has rapidly changed definitions of jobs, work, work organisation and hierarchy and the skills currently recognised as “must have” to be competent and successful. Veterinary medicine, in addition to requirements for expanding veterinary professional competencies, faces the demand to use more both “soft” skills (such as communication, leadership, team work) and competencies from non-medical disciplines (management, governance, ICT, regulatory framework etc.). For sectors such as financing or transportation, with structured systems of operations, predefined variation of mostly mechanistic inputs and influences, and globally harmonised procedures, it is far simpler to integrate ICT. In contrast, the food production and animal health sectors include many animal species and types of production, unfolding in a variety of production systems (i.e. traditional, extensive, and intensive), market economy frameworks (free, centralised or mixed), socio-economic environment (western democracy, post-socialist transitional, developing etc.) all coupled with more complex and diverse biological risks resulting from globalisation, urbanisation and climate change [3]. It cannot be expected that ICT including artificial intelligence (AI) will replace field veterinarians or provide universally applicable tools for all operational aspects of our sector, not withstanding ICT's already growing role in our work and the opportunities it affords to exploit new technologies for our professional and societal affirmation [4].

## **2. Drivers of changes in veterinary operations**

Widening horizons during the second half of the last century in terms of globalisation and international trade had a spill-over effect on animal production and health worldwide, embodied in the Sanitary and Phytosanitary Agreement (SPS) of the World Trade Organisation [5]. Key provisions of the SPS (transparency, harmonisation, risk analysis, equivalence, regionalisation) and strengthening roles of international organisations in standard setting (OIE – World Organisation for Animal Health, IPPC – International Plant Protection Convention and *Codex Alimentarius*) shaped major changes in the perspective and operation of animal health management and food safety on both national and global levels. These changes are reflected in the development of output-based animal health policies focused on prevention and biosecurity, food safety monitoring throughout the supply chain including pre-harvest, and redistribution of responsibility and financing between public and private sectors.

### *2.1. Risk mitigation: biosecurity rather than control/containment*

The purpose of animal health control programs and measures has changed from counting cases and outbreaks and ensuring decreasing trends of disease incidence to demonstrating and maintaining “freedom of disease” at country, industry or farm levels, achieved by active surveillance and biosecurity. According to the European Union (EU) animal health law (2016), biosecurity is one of the

key prevention tools at the disposal of producers and others stakeholders for preventing introduction, development and spread of transmissible animal diseases to, from and within an animal population [6]. Risk assessment, once reserved for assessing disease risk at country and/or regional levels for the purpose of international trade, has become pivotal for effective farm biosecurity, while biosecurity plan implementation and maintenance relies on cost effectiveness (i.e. recognised financial justification by farmer/establishment for disease prevention measures).

Country borders are another location for implementing biosecurity in order to prevent disease introduction. Improving border biosecurity without major disturbances to the movement of people or goods is an on-going challenge, since these movements are one of the major factors promoting economic development, cohesion and market competitiveness.

## *2.2. Integrated food safety/quality control systems*

Sanitary supervision of food on the retail market under jurisdiction of veterinary or public health administration (i.e. inspection) proved to be costly and inefficient as trade and movement of animals and products increased and was liberated. Following food-borne incidents in the United States during 1970s (broken glass found in dry baby food, death and illness due to *Clostridium botulinum*) and the bovine spongiform encephalopathy (BSE) crisis in 1990s in Europe, the need was recognised “to re-establish public confidence in food supply, food science, food laws and food controls” [7]. Integrated food safety and quality control systems have been developed to ensure high levels of public health and food safety, on the level of both individual producers (i.e. GMP – Good Manufacture Practice, GAP – Good Agricultural Practices and HACCP – Hazard Analysis and Critical Control Points) and countries (i.e. “farm to table policy” of the EU). In formulating the “farm to table policy”, the European Commission (EC) wanted to promote defined roles and responsibilities of stakeholders, traceability, transparency, risk analysis and risk management.

Documentation and traceability not only enable reduction of food-borne disease incidents but also aid outbreak investigation. These achievements further support prevention and control measures through identifying critical control points to reduce contamination (hazard and risk analysis). However both traceability and risk analysis work only if quality, consistent and real-time data from veterinary surveillance are available.

## *2.3. Increasing responsibility and initiative of the private sector*

Public policies in developed countries are made with consideration of the interests and participation of those groups who will take part in their implementation and who will carry both burden and benefits of their effects. Such policies have shown to be more accepted, feasible and effective, bringing with shared governance also shared responsibility and distribution of costs among stakeholders. Thus, stakeholders responsible for designing and implementing animal disease surveillance and food safety programs are national and international veterinary authorities, livestock and food/feed industries and farmers’ associations. Farmers are considered responsible for having and consistently implementing biosecurity measures on farms, and food industries and producers are accountable for implementing in-house quality control systems (i.e. HACCP), while the public sector still has a role in promoting, harmonising and optimising health and food safety standards and requirements. Maintaining these partnerships faces challenges of continuously reducing public funding on one hand and on the other, the needs of business for a stable operating environment and reliably foreseen revenues. These business needs are in contrast to the continuous and accelerating surge of new biological and chemical risks and consumers’ demands for rigorous controls.

## **3. Some examples of addressing the demands of drivers by using ICT**

Considering the above-mentioned major drivers of change in veterinary operations worldwide, ICT has already been recognised as providing solutions and has found even wider roles in veterinary medicine. Currently used ICT tools and applications can be categorised according to their purposes as follows:

### 3.1. *Veterinary clinical practice tools*

Veterinary practice management tools available as commercial software incorporate patient and/or practice management. They can be cloud- or web-based and generally include electronic health records, billing and financial analysis. Some tools also accommodate clients with options for on-line (web or smart phone) appointment scheduling, insight into courses of treatment and follow up. Compared to early tools, today, they even incorporate options for electronic clinical audit (quality control protocol) and monitoring antimicrobial usage. Comparably to human medical research, electronic animal patient records are even used for population surveys [8].

In addition to digital and computerised diagnostic equipment commonly used in veterinary practices (i.e. haematology analysers, digital imaging diagnostics), smartphone apps are now available to transform phones into a clinical-quality, single-lead electro-cardiograph (ECG) recorder (partnered with an ECG monitor) [9]. Technological advances and the increased availability of 3D-printing is used in producing 3D models representing patient's anatomy very accurately and applicable in planning surgery, radiotherapy, customised prosthetics and veterinary education. Additionally, bio-printing, the printing of live cells and tissues to be implanted without any autoimmune reaction, is currently an active field of research [10].

### 3.2. *Herd health management tools*

Information management systems for health and production of farm animals were developed in the 80s to support farm management, prevent disease introduction, ensure product quality and optimise output:input ratio and farm development [11]. These tools started with recognition of the need for farm data collection and analysis, due to their potential to improve feeding, disease and reproduction management efficacy. These tools assist both farmers and veterinarians in decision making processes.

Coupled with computerised herd health management, biosensors and biosensing methodologies are now in use for the specific measurement of individual and multiple parameters covering an animal's physiology as well as monitoring of an animal's environment. The nanotechnology approach in developing biosensing tools offers direct benefits through simpler testing, smaller size, greater accuracy, faster results and faster responses to key health threats in the farm animal sector [12].

### 3.3. *Animal health databases and alert notification*

Sharing information *via* new ICTs provides real-time notifications on disease events, enabling successful control of epidemics. For example, Members of the OIE must report within 24 h the occurrence of animal diseases listed by the OIE, the emergence of new diseases and significant epidemiologic events. For this purpose in 2006, the OIE created the World Animal Health Information System (WAHIS) that is coupled with the WAHIS interface and provides information on 117 diseases [13]. The WAHIS interface provides public access to all data as soon as they are validated by the OIE. In addition, the OIE developed their WAHIS-Wild interface that provides information about non OIE-listed diseases in wildlife. Implementation of WAHIS has significantly improved and accelerated the OIE's capacity to relay information about the global animal disease situation, and the member states' awareness of the disease situations in other countries [14]. Due to rapid technological progress, the OIE is working on redesigning and upgrading WAHIS, and a new platform is expected to be completed by the end of 2019. It should make it easier for users to collect and report information and upload data from their own databases, and it will include new features like geospatial data, interconnection with other international or regional information systems, and the WAHIS Alert mobile application.

The EU has developed a similar notification system, the animal disease notification system (ADNS). ADNS is a web-based tool that ensures immediate notification of alerts and detailed information about outbreaks of animal diseases in the member countries, but use by other non-EU countries is enabled on a voluntary basis. Since 2012, a joint project between the EC and the OIE has worked on linking WAHIS and ADNS.

Some other global electronic databases and alert notification systems are RASFF – rapid alert system for food and feed (EFSA – European Food Safety Authority), EMPRES-i – global animal disease information system (FAO – Food and Agriculture Organisation of the United Nations) and PubMed (ISID – International Society for Infectious Diseases), while one database even covers health events of joint interest for humans, animals, plants and the environment (One Health). There are also country-level electronic animal health and production databases for official or public use [15-18].

### *3.4. The animal and animal products trade and veterinary certification*

The EU system of traceability within the food supply chain includes animal identification, certification of shipments and products, and the ICT tool, trade control and expert system (TRACES). TRACES is a web-based multilingual online management tool for all sanitary requirements on intra-EU trade and importation of animals, semen, embryos, food, feed and plants. It was developed after outbreaks of classical swine fever in the EU in 1997 and launched as a compulsory tool for all EU member states on 1 January, 2005 [18]. The main objective of TRACES is to digitise and harmonise certification processes and linked procedures, allowing traceability through monitoring the movements of consignments within the EU and from non-EU countries, facilitating information exchange and improving risk management. This should ensure prompt reactions to health threats. [19]

Electronic animal identification (EID – injectable, ear-tag and bolus transponders) that began emerging in the 1980s is increasingly widespread and is becoming even more inevitable with digitisation of databases [20]. Digital storing and monitoring of data is a norm for in-house quality control systems in food production establishments.

### *3.5. Animal disease data analysis*

The SPS agreement provisions rendered the mandatory use of epidemiological principles in prioritisation, risk assessment and surveillance of animal disease. Since population-based and observational surveys as methodologies used in epidemiological research are founded on biostatistics (i.e. representativeness and quantifiable estimation of parameters, trends, differences and errors), this made them complementary with ICT, and many tools are available to translate formulas, mathematical laws and prediction models into needed solutions. Among the first computer software packages were Epi Info<sup>TM</sup> and EPISCOPE, which covered many epidemiological principles and calculations, and are intended to be used both in teaching of epidemiology and analysis of field data [21, 22]. These products include sample size calculations, statistical comparison and data analysis for different study designs and diagnostic testing protocols. Products are available for data geo-referencing, spatial analysis and epi modelling (i.e. QGIS, R, INTERSPREAD PLUS, OH-SMART), risk analysis (@Risk), web-based epi calculators (i.e. EpiTools) and designing risk-based disease surveillance (i.e. RISKSUR).

Geographic information systems (GIS) are long recognised as an important asset in the field of surveillance and monitoring of animal diseases [23]. GIS functions with geographical data, farm locations and disease information are used in recording and reporting information, epidemic emergencies, cluster analysis, modelling disease spread and planning control strategies. These systems are now coupled with unmanned aerial vehicles (drones) mainly used to acquire real-time data and to constantly update the risk-related information in hotspot areas. The detailed ecological and environmental data they collect can be used for assessing factors (e.g. movement and distribution of people, animals, and pathogen-carrying insects) influencing the transmission of infectious diseases [24].

### *3.6. Tools for veterinary education*

The introduction of problem-based learning into veterinary curriculums expanded the use of ICTs in veterinary education, since these tools offer adaptable, affordable and interactive settings for veterinary students to gain required knowledge [25]. Traditional classroom lectures today almost always include projected presentations that have moved away from one-dimensional presentations

towards immersive, tech-enabled and interactive learning. In addition to Microsoft PowerPoint (for Windows) and Keynote (for iOS), there are numerous other software products for presentations available (i.e. Google slides, Prezi), classroom management software products (i.e. ClassFlow, Insight), and add-on tools for interactive lectures (i.e. Mentimeter, Pool Everywhere).

Spatial computing, which incorporates virtual reality (VR) and augmented reality, has been used in human medical care and education and recently expanded to veterinary education. Examples are the VR project called EZ Anatomy, a realistic anatomy training tool for veterinarians, and also haptic cow and haptic horse (veterinary virtual reality simulators) [26].

Web representation of veterinary faculties worldwide has been expanded to platforms that serve not only for sharing information and administration of programs and courses (i.e. enrolment, exam scheduling, student records), but are increasingly teaching podiums with e-learning and long-distance learning as integral segments of regular coursework.

### *3.7. Animal health diagnostics*

According to the announcement from 2018, a team of scientists led by Brunel University London is working to develop a molecular test and a smartphone app that, when used together, will detect six key pathogens in poultry [27]. The idea behind the project is that farmers collect samples from birds using a matchbox-sized instrument that screens the DNA and RNA. The device connects wirelessly to the app to display the results, which can also feed into a central database and help track outbreaks. The whole process takes less than an hour. Similar examples are already developed for real-time field diagnostics of foot-and-mouth disease [28].

ICT data management systems are now commonly used in laboratories. The laboratory information management system (LIMS) is recognised as a powerful tool in many aspects of laboratory work including harmonisation and completeness of input and output data, laboratory accreditation (ISO IEC 17025), public health surveillance, outbreak investigations, and pandemic preparedness. This system enables active formal checks and controls on business rules and inter/intra automatic data exchange between laboratories [29].

## **4. ICT/AI technologies and trends – demands or opportunities for veterinarians**

Most of the ICT tools used today in veterinary medicine are still strictly related to data collection, analysis, management and dissemination, since the tools have inherit capacity to organise and deal with large volumes of data. As never before, information on new disease outbreaks and/or biological emergencies are shared to national and international authorities, farmers and consumers very rapidly after the disease occurrence. Social networks and other informal, non-governmentally controlled, communication channels globally disseminate information on serious food-borne incidents in tourist resorts even before patients are hospitalised. Using the same resources, our veterinary clients are better informed than ever on health issues of their pets or productivity failures of their farm animals, and are requesting the best quality veterinary services at the cheapest price. The ICT world is putting the veterinary profession under constant and increasing pressure.

Development of AI tools and their application in veterinary medicine, in contrast with some other services and professions, are in the very early phase. A significant amount of animal health, welfare, production and food safety data are already available at aggregated levels (not only country, but regional or even global). Now, though, we are faced with the question of how to merge the data with ICT/AI knowledge and tools for the benefit of our profession and so we can more efficiently respond to requests from clients, industry and society. Though a common concern and perception of all professions prevails that the increasing influence and development of ICT/AI will result in loss of jobs and negatively impact the welfare of personnel, scientists are in agreement that it will be much more difficult to replace humans with machines and software in less routine jobs that demand the simultaneous use of a wide range of skills, and that involve dealing with unforeseen scenarios [30]. How routine is our profession, and what kind of professional development is foreseen in the future? This paper is not intended to answer those and other related, important questions. However, in any

attempt to do that, we need to acknowledge fact that two particularly important non-human abilities that ICT/AI possesses are connectivity and updateability [30], and both of them are highly appreciated and demanded by our veterinary clients today. It seems we veterinarians still rely on the axiom that technology is never deterministic, and the fact that something can be done does not mean it must be done. For sure, it is difficult to predict what sort of impact machine learning and automation will have on different professions in future. However, we must be aware that if the focus of our profession, as it is today, stays on processing information, i.e., collecting suitable data, analysing that data and producing a diagnosis, it might be easily replaced by technology and animal owners. For example, companion animal owners will probably have an AI VET app on their smartphone decades before a reliable technician might be virtualised. As improvements to ICT and AI continue, to interact with these tools, our veterinary profession will need to repeatedly learn new skills and introduce changes.

The reality of change in today's world is axiomatic, but compared to the expanding use of ICT in our field, there is a lack of scientific research that address it, even for systems already in use (i.e. TRACES, EMPRES, ADNS). Our pilot study (not published) identifying farm animal client interest and expectation in ICT-based veterinary consultation platforms found the majority of our clients are active users of ICT resources when seeking information on animal diseases, treatments and market opportunities. However, in the same study, veterinarians showed significant reluctance to use ICT to provide their services to their obviously adaptable clients. Are we to remain only sporadic operators of new technologies and regress into a new era of veterinary science, from being a well-established and necessary profession back to being a vocation doomed to extinction?

Current work on merging biological data, collected *via* biosensors, drones, or other tools with ICT and/or AI platforms and algorithms is expected to accelerate the changes and challenges ahead of us. The specific symbiosis of bio- and information technologies has opened a new era in health and food production. To be controversial, we see this as a chance for all veterinary professionals to evolve from being passive followers of such development to being leaders and collaborators. Significant changes in our education and more interdisciplinary research will be needed to support this projection.

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# Advances in foodborne outbreak investigation and source tracking using whole genome sequencing

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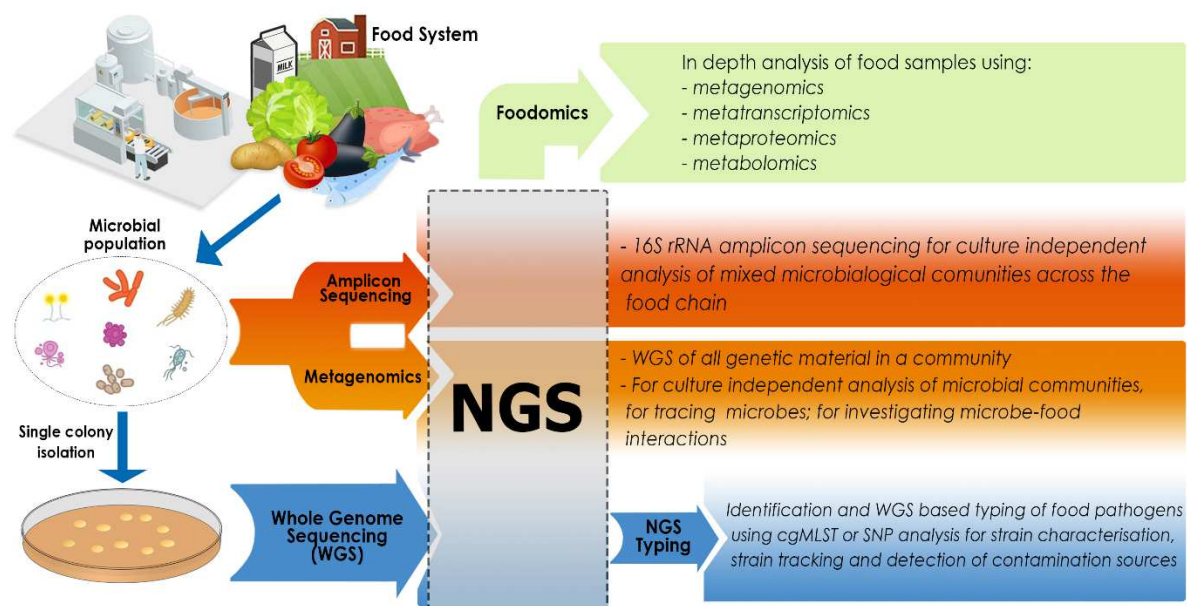
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**Abstract.** The progress in sequencing technology has revolutionized the fields of public health and food microbiology. Today, whole genome sequencing allows high-throughput analysis of entire bacterial genomes at affordable costs. Whole genome sequencing has become a daily routine process for surveillance of foodborne infectious diseases, outbreak investigation, and pathogen source tracking. Several studies on a variety of bacterial species have shown that whole genome sequence-based typing approaches are currently the most powerful typing tools. Whole genome sequencing allows the extraction of information on phylogenetic relatedness, antibiotic resistance, virulence-traits, serotype and multilocus sequence type of an isolate from a single analysis. The optimal typing resolution achievable by whole genome sequencing makes it possible to monitor even small genetic variations occurring in an outbreak strain during the course of an outbreak, making transmission events traceable. Whole genome sequencing allowed the creation of global databases based on standardized nomenclatures like the current multilocus sequence type databases. The benefit of global databases is the international exchange of data as a prerequisite for cross border outbreak investigation, strain tracking, and source identification in the global food chain. With further technological advancement, metagenomic approaches may provide future solutions, allowing complete pathogen detection and characterization directly from specimens.

## 1. Introduction

With increasing industrialization of food production and the international trading of fresh and frozen food, food safety is today a global challenge [1]. The most important aim of food analysis is to ensure the safety and quality of food and to protect consumer from food adulteration [2]. Food associated health problems caused by food contamination or malnutrition have a worldwide impact on public health and economics. Adaption of microorganisms, climate change, modifications of the human life style and demographics, changes in economic development, excessive land use and increasing environmental pollution can cause the emergence of new microbial threats [3]. Food contamination with pathogenic microorganisms and toxins through soil, water and air, and livestock that was fed with contaminated feed, is a major global health threat [4]. Surveillance is an indispensable requirement for early detection of microbial threats, which allows the timely implementation of appropriate measures to terminate outbreaks, and prevent further transmission and morbidity. In our today's global world, where pathogens easily cross national borders, disease monitoring requires efficient local, national and international surveillance systems [3]. The rapid progress in new high-throughput technologies like genomics, transcriptomics, proteomics, and metabolomics has defined the term "foodomics" as a new discipline and increased the standards for food safety [5, 6] (Figure 1). These foodomics tools represent new standards used in foodborne outbreak investigation, in food analysis and in food monitoring from harvesting, processing, transport and storage to final consumption [2].





**Figure 1.** Next generation sequencing applications in food analysis (adapted from [6])

## 2. Whole genome sequencing applications in food microbiology

For detection of pathogenic microorganisms, and for foodborne outbreak investigation and identification of the source of infection, the rapid progress in sequencing technology from Sanger sequencing to whole genome sequencing (WGS) or next generation sequencing (NGS) is revolutionizing the fields of public health and food microbiology [7]. The superiority of WGS-based strain characterization has led to the replacement of former gold standard typing tools like for example fluorescent amplified fragment length polymorphism (fAFLP), pulsed-field gel electrophoresis (PFGE), multiple-locus variable number tandem repeat analysis (MLVA) and serotyping. WGS-based typing based on either single nucleotide variants (SNVs), on gene-by-gene allelic profiling using core genome multilocus sequence typing (cgMLST) or whole genome multilocus sequence typing (wgMLST) is currently the most powerful diagnostic typing tool. The general benefits of WGS-based strain characterization approaches compared to traditional methods are robustness and superior discriminatory power, and the possibility to infer the geographic origin and to obtain evolutionary information for outbreak isolates [8]. The significant decrease of WGS costs allows, nowadays, the broad use of these technologies in daily routine applications in public health and food agencies. In addition, for public health and food laboratories, the high data quality, the reproducibility and accuracy of WGS technology has been demonstrated [9]. For backward compatibility to datasets obtained with traditional methods, information on serotype, classical multilocus sequence type (MLST) or MLVA data can be extracted from WGS data [10]. For public health and food agencies, it is important that both SNP- (single nucleotide polymorphism) and MLST-based approaches yield concordant results for phylogenetic clustering and complement each other [11-13], which allow, in the case of foodborne outbreak investigation, the responsible outbreak source to be identified with a high level of confidence independent of the used analysis pipeline [11]. The resulting information is the basis for correct decisions required in outbreak situations to stop further transmission and to terminate the outbreak [14]. The setup of open accessible databases allows data sharing between public health and food laboratories worldwide and facilitates international source tracking and multinational outbreak investigation [15].

Therefore, the application of WGS in combination with epidemiological analysis provided a new level on the investigation of foodborne pathogens involving *Cronobacter sakazakii* [16], *Listeria monocytogenes* [15, 17-22], *Salmonella* [23-24], Shiga-toxin producing *Escherichia coli* [8, 25-27],

and *Yersinia enterocolitica* [28]. WGS allows the efficient tracking of pathogens entry routes and distribution from farm-to-consumer. Subpopulations of bacterial pathogens can be transmitted from environmental sources outside processing facilities (animals, incoming raw materials, soil, dust and water) into food processing environments. Bacteria can persist in biofilms on stainless steel surfaces, equipment, floors and cold storage areas over long periods. From production facilities they can spread *via* aerosols, personnel, food workflows, and contaminated contact materials to food, and finally, to the consumer [19, 29]. WGS allows the characterization of these subpopulations at each step – from the product, the environment and clinical samples. Since farms and suppliers can have more than one customer, genetically identical bacteria can spread to multiple consumers, distributors or food processing facilities [20, 29].

The high resolution achieved by WGS-based typing applications allows faster identification and more successful investigation of outbreaks when epidemiological information is unavailable [21-22]. Expanding the use of WGS-based typing analysis globally will ensure the rapid implementation of interventions to protect public health, inform risk assessment and facilitate the management of national and international foodborne outbreaks

WGS-based strain characterization is an already established process used in microbiological laboratories in daily routine diagnostics for strain characterization, surveillance, outbreak investigation, and source tracking [14, 16, 19-20, 30]. However, the use of metagenomics, an umbrella term that is generally used for 16S rRNA amplicon sequencing and shotgun metagenome or whole metagenome sequencing, is significantly more challenging and still needs improvements before it can be used as an accurate routine diagnostic tool [31-32]. A critical step in the analysis of metagenomics data are sequence assembly algorithms that must be able to reconstruct genes and organisms from complex mixtures. Therefore, due to the complexity of the entire gene mixture present in a sample, the assembly of metagenomics data is a major bioinformatic challenge [33-34]. However, despite this drawback, the benefit of metagenome sequencing, compared to current microbiological methods is the culture-independent analysis, providing nearly unbiased information on microbial communities from either food materials or the production environment [35-36]. In addition, whole metagenome sequencing allows the extraction of further information relevant to food safety like the occurrence of antimicrobial resistance genes, virulence genes and toxin genes and, when linked to transcriptomics or proteomics data, functional capacities and biochemical activities of microbial populations can be identified (Figure 1). The future improvement of WGS techniques together with the development of new and more accurate bioinformatics tools and pipelines will facilitate the application of WGS technologies on a daily routine basis.

In conclusion, the use of WGS provides several advantages of superior discriminatory power for strain characterization, robustness and stability, which is decisive in cluster detection, backtracking the source and reservoir of the causative strain, when epidemiological information is scarce, and for gaining knowledge on the evolution of emerging pathogenic strains. WGS technologies provide not only benefits for public health and food agencies but also for the food industry throughout the farm-to-fork principle and upcoming improvements in technology and bioinformatics with the perspective of metagenomic sequencing applied directly to the sample specimen.

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# Food safety and environmental risks based on meat and dairy consumption surveys

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**Abstract.** This paper gives an overview of the possibilities of using meat and dairy consumption studies in food safety and environmental risk scenarios. For both types of risk-based scenarios, common denominators are consumption patterns such as frequency and quantity of consumed food, demographic profile of consumers and food safety hazard or environmental impact of a specific type of food. This type of data enables development of simulation models where the Monte Carlo method is considered as a useful mathematical tool. Synergy of three dimensions – field research used in consumption studies, advanced chemometric tools necessary for quantifying chemical food safety hazards or environmental impacts and simulation models – has the potential to adapt datasets from various sources into useful food safety and/or environmental information.

## 1. Introduction

Knowledge of food patterns of a certain population is important for understanding various dietary profiles [1] and can aid in deploying the data into various risk-based scenarios. In order to perform a food consumption survey, it is mandatory to develop a structured questionnaire taking into account general principles and guidelines on data collection of national food consumption outlined by the European Food Safety Authority [2]. It covers basic demographic data such as gender, age and weight, and depends on the type of research specific information such as frequency and quantity of food consumption [3].

The Food and Agriculture Organization/World Health Organization (FAO/WHO) define exposure assessment as a “qualitative and/or quantitative evaluation of the likely intake of a chemical agent via food” [4]. To perform such an analysis, contamination and food consumption data are combined to obtain an estimation of the exposure level.

Azzura *et al.* [5] recognise food as one of three consumption domains with the largest environmental impact share. Food consumption is associated with water pollution, climate impact and loss of biodiversity [6]. This brings us to the transition of food chains towards developing sustainable food systems [7]. In order to analyse environmental impacts associated with food consumption, it is important to have dietary patterns and consumed quantities.

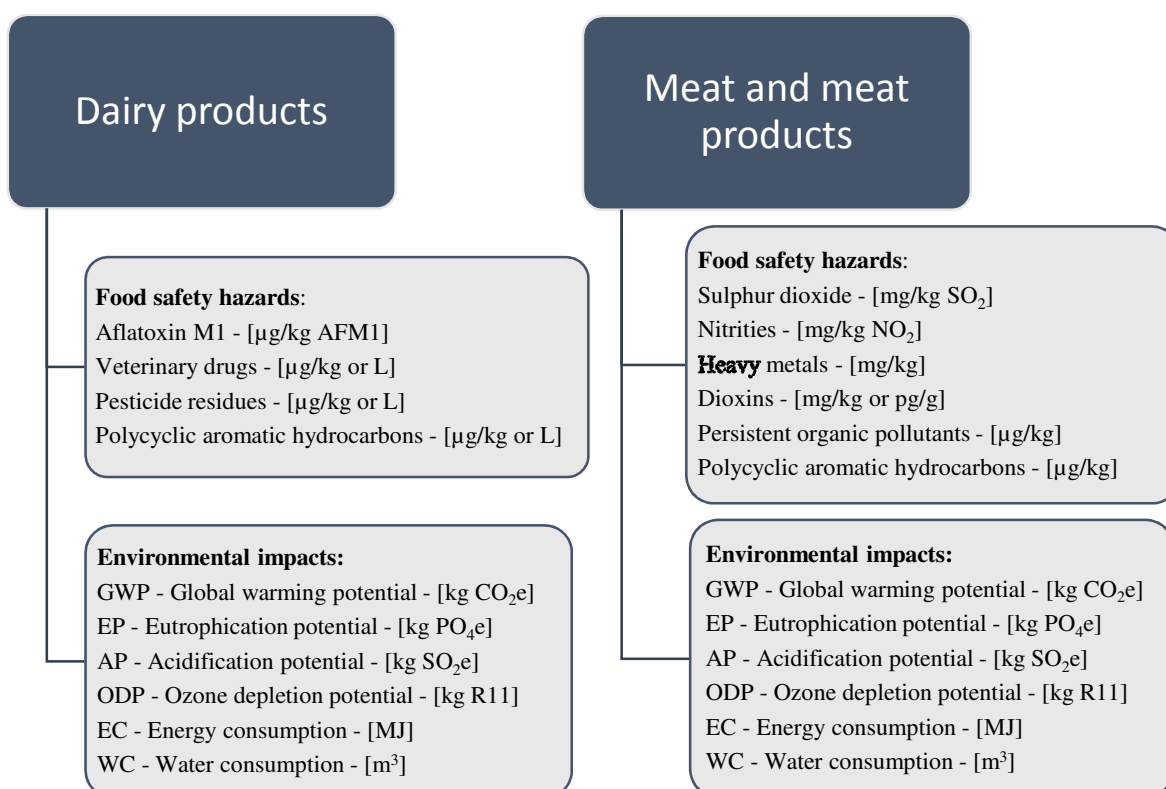
The aim of this paper is to present a generic food safety and environmental risk-based model based on consumption surveys dealing with animal origin food.





## 2. Materials and methods

For the purpose of this paper, a literature review was performed through examining scientific papers covering food safety and/or environmental risks of animal origin food spanning the research for the period 2000 - 2018. The emphasis was on international journals to assure appropriate scientific content, and selection of manuscripts was based on the journals' impact factors and preferring those indexed by international repositories such as Scopus index and Web of Science. This research identified that the majority of publications covered quantification/modelling of two types of risks – food safety and/or environment deployed on specific types of food (dairy products, meat and meat products, poultry, etc.). Figure 1 depicts a brief overview of the analysed chemical food safety hazards/environmental impacts that were in focus [8-28].

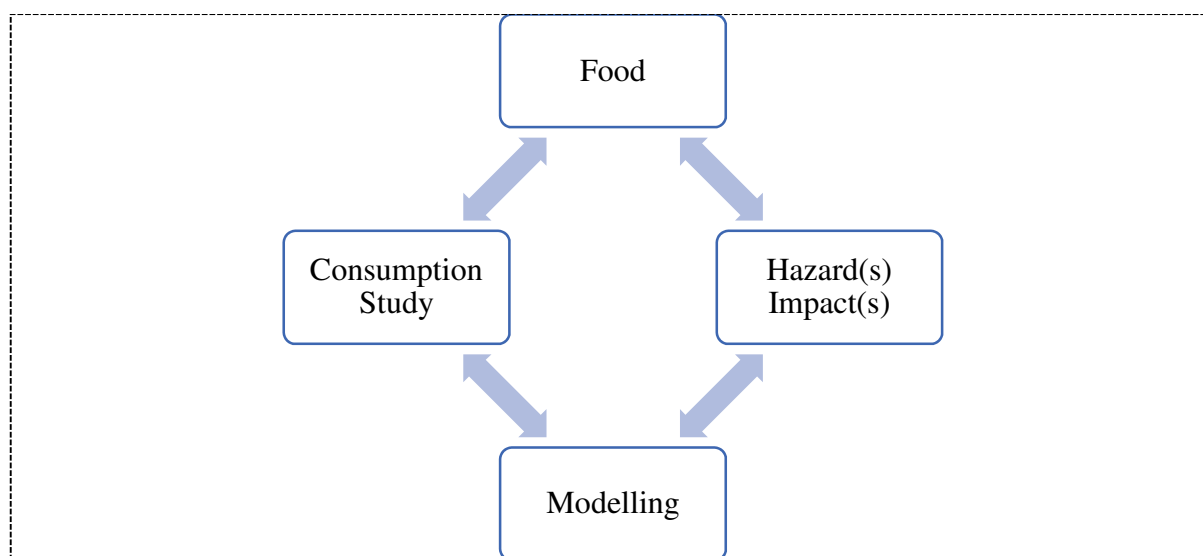


**Figure 1.** Overview of analysed chemical food safety hazards and environmental impacts

## 3. Modelling consumption studies

A generic food safety and environmental risk-based model based on consumption surveys dealing with animal origin food is presented in Figure 2. It consists of the following four elements: (i) Food; (ii) Consumption study; (iii) Food safety hazard and/or environmental impact; (iv) Modelling.

Within the first part, it is important to identify product(s) in focus, meaning the study can be performed for one specific animal origin food (yoghurt) or for a group of products (fermented meat products). The second element is related to designing the consumption study in terms of sample size, sampling methods and type of questionnaire to be used. The third element is associated with defining food safety hazard(s) and/or environmental impact(s). Within this element, it is important to define how the data will be obtained, i.e. from experiments, from expertise, or from literature review. Finally, the fourth element is modelling the data, identifying equations to be used and mathematical simulation to be employed, bearing in mind uncertainties linked with the quality of data.



**Figure 2.** Generic food safety and environmental risk-based model

### 3.1. Sample and sampling method

The tested population should be a convenient sample having, as a “rule of the thumb”, at least 500 respondents for a country the size of Serbia with around seven million citizens [29]. With a confidence level of 95% and confidence interval of 5%, estimated sample size should be at least 385 [30, 31]. However, to make the survey comparable to related published surveys, 1,000 respondents should be the right number if we analyse various exposure assessments in the EU ranging from 303 in Cyprus up to 10,419 in Germany [32]. When it comes to age, taking into account that substantial life-changing transitions and changes in eating behaviour usually occur when young adults complete high school [33], it is typical to interview a population over 20 years of age. Respondents are recruited either by randomly choosing citizens near food retailers or by employing existing professional and family networks, and further dissemination of the questionnaire through their networks [34].

Finally, the recall periods differ, but two of them prevail – the 24-h dietary recall as the most common recall method used or a 7-day dietary recall [2]. EFSA suggests that in some surveys, it is more efficient to include more recording days per person in order to estimate habitual exposure to compounds from foods.

### 3.2. Questionnaire – research instrument

Since these studies are about consumption quantities, the questionnaire should have understandable questions related to the frequencies and amounts of eaten food. To avoid any bias in self-reporting, it is not unusual to place food on plates/dishes (as they are usually served) and determine exact values of product portions (in grams or mL) and take photos of the investigated food [34]. Then, the interviewees are provided with photographs as a visual aid including the weight/volume of the portions so they can exactly report their consumption patterns.

### 3.3. Modelling food safety risks

Human exposure to a certain contaminant through consumption of food can be calculated using consumption data for animal origin products, concentration of a certain contaminant in animal origin food and body weight, as follows [35]:

$$EDI = \frac{\sum_{i=1}^n Q_i}{d} * \frac{1}{bw} * C_t \quad \{1\}$$

EDI is the estimated daily intake of a certain contaminant [ $\mu\text{g/kg bw/day}$ ].  $Q_i$  is the quantity of animal origin food consumed [kg]. Average daily intake of animal origin food is divided by the number of recall-days,  $d$ . Body weight ( $bw$ ) is expressed in [kg].  $C_t$  is the concentration of contaminants [ $\mu\text{g/kg}$ ].

Regarding concentration of contaminants, the use of raw data is recommended in order to assume the statistical distribution of the data. If not, then results from other studies can be used, as was presented in exposure assessments of aflatoxin intake through consumption of maize [34] or dairy products [3], where recent publications were used to determine concentration limits. However, if it is not possible to assume data distributions, and if the number of analysed samples is low with many data below limit of detection, triangular distribution can be assumed [3, 36].

If necessary, equation {1} could be expanded by adding a coefficient reflecting the content of animal origin food (i.e. content of meat in a meat product, content of milk in a dairy product, etc.). However, it is important to note that content should be calculated based on food in the “ready to eat” or “ready to serve” form.

Mathematical simulation has become an essential tool in exposure assessments using software to recreate scenarios, like consumption patterns [37]. Our literature review revealed that Monte Carlo simulation is one of the techniques often used in analysing chronic exposure assessment scenarios, such as the works of Wang *et al.* [38] related to health risk assessment of Chinese consumers to nickel or Cardoso *et al.* [39], covering methyl-mercury intake through cephalopods in Portugal. Monte Carlo simulation assumes a particular distribution based on the data and involves the use of random numbers to perform a stochastic simulation and, therefore, is recognised as a powerful tool to analyse complex problems that can occur in various food safety scenarios [37]. In order to estimate the intake of a certain contaminant by the entire population, it is common to perform 10,000-100,000 iterations in Monte Carlo simulations [3]. Therefore, to complete such simulations, it is mandatory to determine the probability distributions for both body weight and daily/weekly consumption patterns [34]. If no probability distribution is supplied or calculated, when comparing data with different distributions (usually provided in statistical software), visual analysis of the distributions should be considered as a technique to assess the fitting of the probability distributions [40].

Data can be further deployed in risk characterisation using exposure levels of diets and contamination levels in foods to predict death and mortality [41]. FAO/WHO and EFSA propose various models for performing risk characterisation [42-45]. An example of risk characterisation of aflatoxin M1 (AFM1) intake through consumption of milk and yoghurt was presented by Udovicki *et al.* [3]. The following assumptions were applied: (i) aflatoxin B1 (AFB1) carcinogenic potency is based on the synergistic hepato-carcinogenic effects of AFB1 and hepatitis B virus infection; (ii) as AFM1 is a metabolite of AFB1, than AFM1 induces liver cancer by a similar mechanism; (iii) the potency of AFM1 is one-tenth of AFB1 [46]. Thus, the carcinogenic potency of AFM1 was estimated to be 0.001 cancer cases/year/ $10^5$  individuals per  $1 \text{ ng kg}^{-1} \text{ bw day}^{-1}$  in Hepatitis B virus surface antigen negative (HBsAg<sup>-</sup>) individuals and 0.03 cancer cases/year/ $10^5$  individuals per  $1 \text{ ng kg}^{-1} \text{ bw day}^{-1}$  in HBsAg<sup>+</sup> individuals [47]. In line with the assumptions, the following equation was applied [3]:

$$P_{\text{cancer}} = 0.001 \times \%HBsAg^{-} + 0.03 \times \%HBsAg^{+} \quad \{2\}$$

As a result, the risk of hepatocellular carcinoma (HCC) incidence per year, resulting from dietary AFM1 intake through milk consumption, can be calculated using EDI data multiplied by the AFM1 cancer potency:

$$HCC \text{ risk} = EDI \times P_{\text{cancer}} \quad \{3\}$$

Additional risk characterisation can be deployed in terms of calculating the margin of exposure (MOE). In order to assume MOE, the use of benchmark dose (BMD), i.e., the dose that causes a low but measurable response or BMDL10 (benchmark dose lower confidence limit 10%), which is an estimate of the lowest dose that is 95% certain to cause no more than 10% cancer incidence, is recommended [42]. The MOE is the ratio between the reference dose and the EDI, and considering overall uncertainties in the interpretation, MOEs equal to or higher than 10,000 would be of little concern from a public health point of view [3].

### 3.4. Modelling environmental risks

Calculation of the environmental impact related to consumption of animal origin food requires a partial life-cycle assessment (LCA) be conducted. As a minimum, system boundaries should cover three subsystems: Farm, Plant and Consumer. Subsystem 1 – Farm should cover all livestock activities; subsystem 2 – Plant includes all food processing activities, while; subsystem 3 – Consumer contains of all consumption activities from purchasing food to discarding food waste [19, 48]. Finally, it is necessary to choose a functional unit (FU) in which the impacts are expressed and which is used as a basis for comparisons [49]. In the meat chain, the most common FUs are one kg of livestock [50, 51]; one kg of carcass [52, 53], and; one kg of meat/meat products [54]. In the dairy sector FU is either 1 L of raw milk or 1 kg of dairy product [19].

The environmental impact caused by consumption of animal origin food can be calculated using data on food consumption, environmental impact of the products calculated from food production (subsystems 1 and 2) and body weight (bw), as follows:

$$EDEI = \frac{\sum_{i=1}^n Q_i}{d} * \frac{1}{bw} * I_e \quad \{4\}$$

EDEI is the estimated daily environmental impact. The latest research confirms that global warming potential (GWP) is often used in presenting the environmental impact of the meat and dairy chains [19, 55]. So, to calculate GWP, EDEI is expressed as CO<sub>2</sub> emissions [kg CO<sub>2</sub>/kg bw/day]. Q<sub>i</sub> is the quantity of animal origin food consumed [kg]. Average daily intake of animal origin food is divided by the number of recall-days, d. Body weight (bw) is expressed in [kg]. The last coefficient, I<sub>e</sub>, is the environmental impact per functional unit (in the case of GWP, this is kg CO<sub>2</sub>/kg). The GWP of the meat chain can be calculated as follows [56]:

$$GWP = \sum_i^n GWP_i \times m_i \text{ [kgCO}_{2e}] \quad \{5\}$$

Where: m<sub>i</sub> is the mass of emitted gas (kg) and GWP<sub>i</sub> is the global warming potential of the emitted gas. The GWP is calculated for every subsystem within the meat chain. The same approach can be applied to the dairy chain.

The importance of analysing the entire food chain is presented in the work of Skunca *et al.* [16], where chicken meat was the focus. The LCA model included five poultry chain subsystems: farms, slaughterhouses, meat processors, retailers and households, and the results revealed that GWP of the farms is equal to the GWP of the other four subsystems combined, highlighting the impact of households/consumers. From a consumer point of view, this study covered purchasing of chicken meat, its storage and preparation, ending with food waste and packaging disposal. Schanes *et al.* [57] points out several activities that directly affect food waste such as planning, shopping, storing, cooking, eating and managing leftovers. How data from households/consumers can be further modelled is presented in an environmental study on household waste in Serbia [58], where GWP was at the focus of a Monte Carlo simulation. Quantities of food waste were calculated using data from a household survey, as follows:

$$QFW_i = \sum_{j=1}^n F_i * Q_i \quad \{6\}$$

QFW is the quantity of food waste [kg].  $F_i$  is the reported weekly disposal frequency of a specific food category,  $i$ .  $Q_i$  is the quantity of a specific food category reported by each of the respondents,  $j$ . The GWP of food waste in terms of CO<sub>2</sub> emitted was then calculated using the data from the survey, as follows:

$$GWP_{FW} = \sum_{i=1}^n FW_i * GWP \text{ [kg CO}_2\text{e]} \quad \{7\}$$

$FW_i$  is the amount of food waste of a specific food category discarded weekly [kg]. GWP is the assumed CO<sub>2</sub> emitted from the food waste. This Monte Carlo simulation quantified the GWP of food waste as being around 3.46 kg CO<sub>2</sub>e/household per week, enabling us to estimate the annual CO<sub>2</sub> emissions due to food waste from Serbian households amounts to 687,346 tons [58].

#### 4. Conclusion

To keep pace with the increased need for simulation models to predict various risks, consumption studies are now an essential part of the process. Synergetic effects of field research needed for consumption studies, joined with advanced chemometric tools and simulation models can help us adapt detailed datasets obtained from different sources and complex samples into useful food safety and/or environmental information. The advantages of this approach enable using one consumption study for different purposes in terms of calculating various food safety and environmental risks. Proposed model, although generic, may be employed in combining food safety and environmental issues.

Future research should explore possibilities of data modelling when dealing with imperfect data, especially when data are from heterogeneous data sources. This is pronounced when modelling activities cover integrating data with various levels of precision and certainty, quantitative or qualitative, different structuration and terminologies. Also, future research should validate models, regardless of type of food (meat/dairy) and physical properties (solid/liquid).

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# Novel strategies and tools for microbial risk assessment of foods of animal origin

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**Abstract.** Risk assessment aims at providing structured information for decision making, public health improvement, regulatory actions and research initiatives. The four very distinct steps in the risk assessment process enable risk management and risk communication, and thereby, a functional food safety system. Identification, characterization and assessment of risks demand the application of science-based, accurate and reliable methodologies. Nowadays, several different widely recognized approaches to risk assessment are applied worldwide. Novel omics technologies are benchmarking a new era of pathogen testing, providing much more than just accurate identification. These technologies have now opened the door for a more integrated approach that can enlighten transmission patterns and predictions of the transmission routes. Merging data on virulence, interaction of pathogens with different food matrices and the host, multiple data processing is resulting in reliable and science-based responses to the forthcoming challenges.

## 1. Introduction

Food culture is a universal language, a primary form of cultural expression that joins people together. Food research should always something for more than just profit, since food has a significant impact on human health, economic growth and world sustainability. Being a strong pillar of the global economy, the food production sector still needs significant improvements through increasing the efficiency and competitiveness of food production companies, primarily through application of new science-based practices and contemporary achievements in this area that would boost innovations required for economic growth and prosperity.

Food safety, being an ultimate priority in the field of food production, demands accurate and reliable models for the assessment and analysis of foodborne risks of hazards to human health and effective control system in place. Following Regulation EC No. 178/2002 of the European Parliament [17], it is clear that food safety risk analysis should be based on the following principles: risk management, risk assessment and risk communication.

Microbiological hazards can cause outbreaks of foodborne illness. On a large scale, these food incidents can cause numerous illnesses, hospitalizations, and chronic medical conditions and can have significant mortality rates. Microbiological risk assessment (MRA) minimises the risks of foodborne hazards that could adverse events among the population. This complex area, as defined by the Codex Alimentarius Commission [12] can be properly monitored if the following activities are undertaken: hazard identification, hazard characterization, exposure assessment and risk characterization. As evidenced so far in numerous epidemiological surveys and foodborne illness outbreak reports, the foodborne disease burden is constantly growing. Therefore, it is necessary to develop comprehensive tools for assessment of the hazards, their proper identification and quantification, determination of their occurrence probability and prioritisation of microbiological risks on the basis of country-specific uncertainties.



The EU and other developed countries worldwide apply MRA strategies to enable comprehensive evaluation of risks that influence the shaping of the food safety strategies globally. Due to the high genetic diversity of microorganisms, it is still a challenge to encompass all the niches of their occurrence, transmission patterns along the food chain and impact on human health. MRA, being a scientific and analytical tool, encompasses the analysis of all the steps in the food production chain (from collection of raw materials, through processing, to the different food consumption patterns, retail outlets, restaurants and private homes). MRA is necessary, and to be effective, it demands science-based data collection, analysis and processing which then lead to valid decision making.

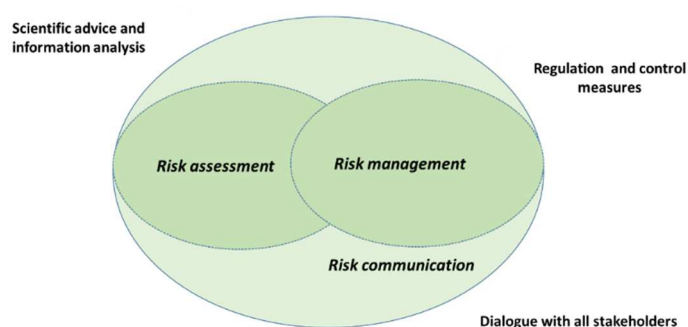
Traditional microbiological methods have long been applied in food safety control, investigation of food-related illnesses and scientific research in this field [11, 41, 44]. However, new technologies are increasingly available that ensure rapid or early detection of precise signals indicating the presence of microbiological hazards in foods [11, 10, 28, 41]. In the digital era, the implementation of new reporting models is mandatory for improving the detection of food-related hazards (and evaluating their risks). For this reason, analysis of population-based studies is needed to provide science-based knowledge of foodborne pathogens.

Rapid detection of foodborne pathogens, understanding their transmission routes and their relation to environmental conditions, monitoring trends, and understanding antimicrobial resistance and prioritisation are of the utmost importance for food safety. Therefore, health authorities worldwide have established comprehensive programs for the purposes of surveillance and management of effective food safety systems.

Nowadays, whole genome sequencing (WGS), a relatively new technique, provides the most reliable tool for typing of foodborne pathogens. Not yet widely applied, its potential indicates it will be a primary choice for accurate and reliable implementation of MRA.

## 2. Risk analysis paradigm

A broader paradigm of risk analysis includes three important components [12] as depicted in Figure 1.



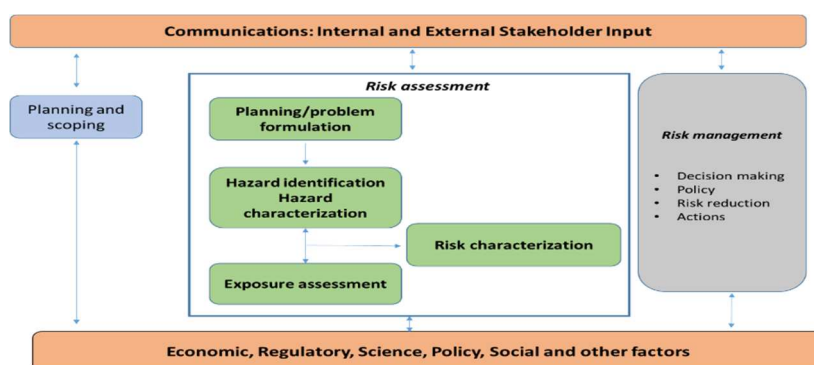
**Figure 1.** The components of risk analysis

*Risk management* is the process of weighing policy alternatives in consultation with all interested parties, considering risk assessment and other factors relevant for the health protection of consumers and for the promotion of fair-trade practices, and, if needed, selecting appropriate prevention and control options.

*Risk communication* is the interactive exchange of information and opinions throughout the risk analysis process concerning risk, risk-related factors and risk perceptions, among risk assessors, risk

managers, consumers, industry, the academic community and other interested parties, including the explanation of risk assessment findings and the basis of risk management decisions.

*Risk assessment*, recognized as a reliable tool for providing information necessary for setting up regulations, programs and research in the area of food safety, according to the Codex Alimentarius Commission [12,13] has the following steps: (a) hazard identification; (b) hazard characterization; (c) exposure assessment and (d) risk characterisation. MRA is a science-based approach applied to estimate the probability of exposure to a microbial hazard [12, 13, 18, 19, 21, 22, 23]. It also encompasses scoping, planning, decision making within the frame of risk management, communication with relevant stakeholders, etc. (Fig. 2).



**Figure 2.** Risk assessment framework and its relationship with other components of risk analysis (adapted and modified from [37])

The widely used hazard-based approach, when adequately applied, has the purpose of preventing or reducing pathogenic microorganisms in the food production chain. However, since the zero-risk approach cannot be applied to microbial agents, the regulatory microbiological criteria define criteria for the acceptability of food products or food production processes.

The Codex Alimentarius Procedural Manual, 25<sup>th</sup> edition [13] states that the *Hazard identification* is the “qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food”. Hazard identification is a qualitative process in identifying chemical, biological or physical hazards, their description, impact on human health and mechanisms of action [33].

*Hazard characterization* is defined in the as “the qualitative and/or quantitative evaluation of the nature of the adverse health effects associated with biological, chemical and physical agents which may be present in food” [13]. When applied to MRA, hazard characterisation is the qualitative or quantitative defining of the different effects that a microorganism or its toxin in food can cause. For such definition, different sources of information are used, such are clinical or epidemiological information, mathematical modelling, dose-response models, etc. [13]

*Exposure assessment* is defined as “the qualitative and/or quantitative evaluation of the likely intake of biological, chemical and physical agents via food as well as exposures from other sources if relevant” [13]. This is actual or anticipated exposure to microbial pathogens or their toxins, and the most useful tools for exposure assessment are predictive models. Exposure assessment must include frequency of food contamination by the hazard, ecology of the specific food, possible contamination of the raw material, processing, packaging, and storage and distribution pathways, and the final preparation of the given food.

*Risk characterisation* is defined as “the qualitative and/or quantitative estimation, including attendant uncertainties, of the probability of occurrence and severity of known or potential adverse health effects in a given population based on hazard identification, hazard characterisation and

exposure assessment” [13]. Risk characterisation is actually merged information obtained through previous steps. It estimates the degree of the risk of a hazard in a specified food for a population group.

MRA can be classified in two general categories, quantitative and qualitative. Qualitative risk assessment is mainly descriptive and estimates the risk and factors affecting the risk. This type of risk assessment covers an overview of the literature and available scientific knowledge and uses a systematic approach even though it does not include numerical parameters [4, 18, 19, 24]. It is usually implemented before quantitative assessment [24, 35].

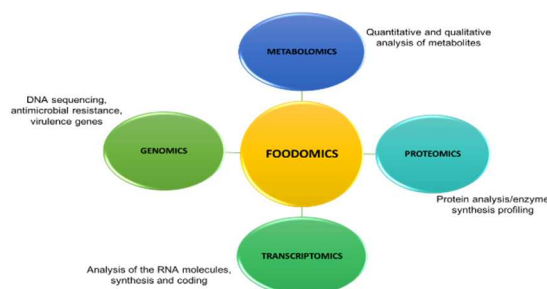
Quantitative microbiological risk assessment generally uses two types of models: deterministic or stochastic. Unlike deterministic models, stochastic models can use estimation of randomness (when defined) and therefore might be a better choice for natural systems. For deterministic assessments, all variables are assigned a certain fixed value, which could be a mean or maximum value or a “worst-case scenario” from a variable data set, for example [4, 18, 19].

Numerous adverse incidents connected to meat production and processing (Bovine Spongiform Encephalopathy (BSE), avian flu, foot and mouth disease, some emerging and/or evolving pathogenic bacteria such as *Escherichia coli* O157:H7 and *Listeria monocytogenes*) have been in the focus of all the stakeholders [27, 42, 43]. Even given existing global regulations and awareness concerning animal production, there are still many arguments confirming that this chain still poses a big threat to public health. Microbiological risks are of the biggest concern in the meat production sector. The possible sources of contamination occur initially at the animal husbandry level, but at all other stages too, including raw meat, through all the steps of production, biofilm formation, storage and distribution. The hazards in food of animal origin can be intrinsic to the live animal or are introduced during handling and processing. These hazards can be classified into zoonoses or environmental hazards. Therefore, application of efficient and accurate methodologies, enabling fast predictive matrices will be in the focus of the attention when MRA for food of animal origin is conducted.

### 3. Novel methodologies used for microbial risk assessment

In the last five decades, a significant improvement has been evidenced in the field of food safety through application of novel, accurate and reliable techniques [10, 7, 11, 28, 44]. Novel technology has induced the inevitable shift from classical microbiological testing to rapid detection methods based on PCR and the most recent omics technologies.

The application of novel omics techniques in MRA addresses the challenges. Recent initiatives in this area were discussed by different stakeholders at the European Symposium of Food Safety, Cardiff, April 20–22, 2015, organized by the International Association for Food Protection (IAFP), while the IAFP European Symposium in Athens, May 11–13, a workshop on Next Generation MRA (Microbiological Risk Assessment) - Integration of Omics Data into Assessment was co-organized by ILSI Europe, IAFP and ICFMH [10, 11].



**Figure 3.** Different applications of omics techniques in the analysis of food pathogens

The application of new technological developments (known as omics) significantly improved general understating of microbial patterns, physiological triggers and behaviour of microorganisms in different food matrices (Figure 3). Among these techniques, the most important ones are genome sequencing, protein analyses and assessment of the metabolic profiles of microorganisms [11, 48]. However, until recently, these technologies were applied solely for scientific research purposes, and not for risk assessments in the area of food safety. Recent studies showed they could be powerful tools in this area [14, 30, 42].

Structuring and analysis of omics data is rather complex, making it difficult to successfully incorporate them into the current MRA paradigm. Rather than focusing on genotypic characterization, the solution to this problem might be the proper analysis of gene expression [6, 10].

Identification of the transmission patterns and origin of diseases can be revealed by the application of Next Generation Sequencing (NGS) techniques, also termed WGS [10, 31, 41, 45]. The technique can immediately offer completely accurate strain-level identification of pathogens, and information on virulence potential and antibiotic resistance that is now competitive with several current methods that must be coupled together to produce the same result [8, 15, 16, 27]. Furthermore, WGS provides information on genes, mobile genetic elements, and horizontal gene transfer [34].

WGS is applied to detection of food pathogens in the United Kingdom, Denmark, France and the United States [2, 3, 27, 32, 35]. In the US, WGS techniques to detect listeriosis outbreaks can more efficiently identify the causative agent at smaller concentrations than traditional microbiological approaches, showing the good potential of WGS for further larger-scale applications [32]. The potential of WGS lies in the fact that, if properly designed and applied, it can replace numerous time-consuming techniques such as serotyping, virulence profiling, antimicrobial resistance, sequencing, etc. [25].

Detection of the presence of foodborne pathogens in a food matrix can be impaired by their low numbers in comparison to the remainder of the microbiota present in the food matrix [9, 20]. However, another possible option for successful employment of the WGS techniques is to focus on identification of pathogens' virulence genes or bacterial interactions and their impact on food safety [9, 11, 20, 26, 47].

Predictive Metagenomic Profiling (PMP) can reveal the functional genes in microbial communities, resulting in prediction of their contribution to risk [11, 46], which can be applied to support MRA studies. Genome Scale Metabolic Models (GSMMs) can be appropriate for detection of specific pathogens [5]. This technique can reveal important information about gene and protein patterns and metabolism of microorganisms [39] and, therefore, can be useful as a predictive tool. This relates especially to the specific components of MRA such as hazard identification and exposure assessment [1, 5, 36, 38].

These novel techniques have limitations, such as their validation for purposes of quantitative risk assessment as well as the lack of standardization of these methods that can impair reproducibility result [40].

#### 4. Conclusion

There is a constant need for improvement of the accuracy and reliability of the current food safety system and the tools for MRA, a strong pillar of food safety. Following on from implementing methodologies and strategies for MRA, novel omics techniques, especially WGS, are a promising area for collection and analysis of vast data on pathogens, their characteristics, and their relation to food matrices and the human population. These technologies should open a new era of fast, reliable, science-based information on foodborne pathogens, validated protocols and improvement of fundamental science. Food safety and microbial modelling should both advance.

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# A path towards modernisation of meat safety assurance in European abattoirs

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**Abstract.** Carcass meat safety mainly relies on official meat inspection and abattoir process hygiene. The deficiencies of the traditional meat safety system are well known. The European Food Safety Authority has proposed a modern, risk-based meat safety assurance system. The process of implementation of the new system in Europe is in the initial phase and is associated with legislation changes. Several challenges are experienced in the implementation process so far and future challenges are anticipated. Further modernisation of the meat safety assurance system and its full practical implementation need to go in tandem with intensive research and training.

## 1. Background

Meat safety at the abattoir level of the meat chain is essentially and traditionally assured through official veterinary meat inspection and abattoir process hygiene. Veterinary meat inspection targets hazards that cause clinical signs and/or detectable lesions in animals at slaughter and gave an invaluable contribution to public health protection from zoonoses through the last century. However, it suffers from many flaws – the main one is that it is not risk-based and does not guarantee public health protection in regard to the most frequent meat-borne diseases today [1]. Abattoir process hygiene relies on prerequisite programmes and HACCP-based procedures and primarily aims to prevent faecal contamination of carcass meat associated with hazards that can only be detected through laboratory analyses. Therefore, abattoir process hygiene and meat inspection are complemented by laboratory testing, which also has many drawbacks including delayed results, non-proactiveness, relatedness only to the hazard examined, expensiveness and limited tests' sensitivities and specificities [2].

The deficiencies of the traditional meat safety system are well known for decades. There is a trend in the European Union (EU) and developed countries worldwide to accomplish meat safety goals with a new, comprehensive concept that considers the occurrence of each hazard in the whole meat chain as a function of the hazard's consequences for public health. The EU General Food Law [3] introduced an integrative farm-to-fork approach to food safety and application of the principles of risk analysis. The resultant "hygiene package" of legislation supported a risk-based approach to meat safety, primarily through Regulation 854/2004 [4]. A decade ago, the European Commission triggered scientific work to support the risk- and food chain-approach to meat safety assurance by delegating the European Food Safety Authority (EFSA) to prioritise biological and chemical public health hazards and recommend the new system of meat inspection and meat safety assurance.



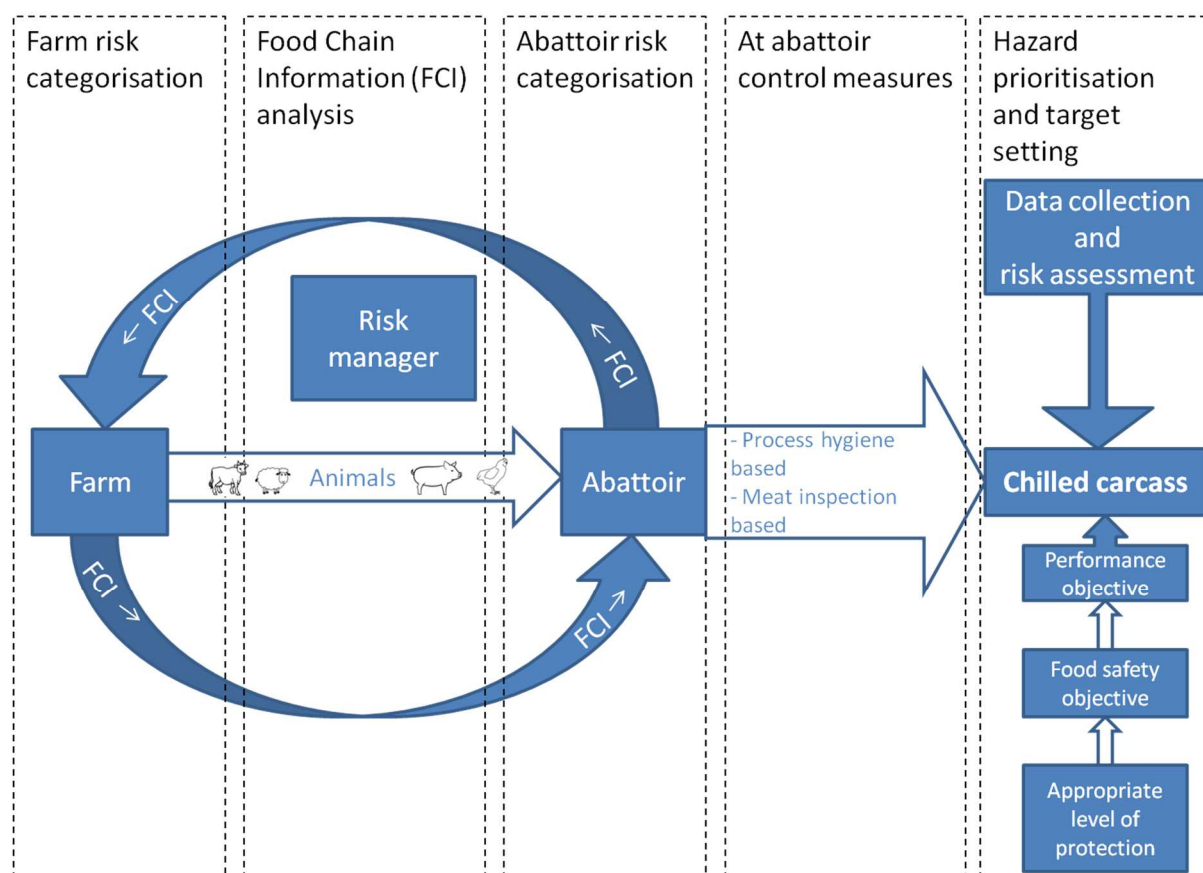
## 2. State of the art

The EFSA's opinions on hazards to be covered by meat inspection [5-10] pose the basis of risk-based meat safety assurance in Europe. The opinions primarily deal with the priority public health hazards, but also with animal health and welfare issues of the new, changed meat inspection practices. The EFSA ranked biological hazards mainly on the basis of incidence and severity of human disease and the strength of evidence that meat from respective animal species is an important risk factor for disease in humans. For chemical hazards, risk ranking was conducted based on the results of the national residue control plans and other specific parameters such as the toxicological profile and the likelihood of the occurrence of residues/contaminants in each animal species. Table 1 shows current priority (increased risk) hazards that originate from slaughtered animals and significantly affect carcass meat safety [5-10].

**Table 1.** Prioritised meat-borne hazards [5-10]

Species	Biological hazards	Chemical hazards
Pigs	<i>Salmonella enterica</i>	dioxins
	<i>Yersinia enterocolitica</i>	dioxin-like polychlorinated biphenyls
	<i>Toxoplasma gondii</i>	chloramphenicol
	<i>Trichinella</i> spp.	
Poultry	<i>Campylobacter</i> spp.	dioxins
	<i>Salmonella enterica</i>	dioxin-like polychlorinated biphenyls
	ESBL-AmpC gene-carrying bacteria	chloramphenicol
		nitrofurans
		nitroimidazoles
Cattle	pathogenic <i>Escherichia coli</i>	dioxins
	<i>Salmonella enterica</i>	dioxin-like polychlorinated biphenyls
Sheep and goats	pathogenic <i>Escherichia coli</i>	dioxins
	<i>Toxoplasma gondii</i>	dioxin-like polychlorinated biphenyls
Horses	<i>Trichinella</i> spp.	phenylbutazone
		cadmium
Farmed game	deer <i>Toxoplasma gondii</i>	none
	wild boar <i>Salmonella enterica</i>	none
	<i>Toxoplasma gondii</i>	
	other none	none

The generic framework of the new meat safety assurance system is presented in Figure 1. The system is supposed to be risk-based, longitudinally integrated and focused on safety of chilled carcasses in abattoirs, primarily in regard to the priority hazards (Table 1). The meat safety levels (targets) of priority hazards in chilled carcasses are intended to be set through appropriate level of protection and related food safety objectives. The targets will be accomplished by the risk manager who will adjust control options in the meat chain, e.g. by the Food Chain Information (FCI) analyses and balance between farms and abattoirs based on their risk categorisations, by setting the intensity of meat inspection procedures (i.e. visual-only inspection of low-risk animals, or more stringent inspection procedures for high-risk animals) and by decision on additional carcass treatments in the case of high risk farms and/or abattoirs.



**Figure 1.** Generic framework of the new meat safety assurance system [based on 5-7]

The legislation changes are a cornerstone of practical implementation of the new system and several changes have already been adopted in the EU legislation. These include process hygiene criteria for *Salmonella* on pork carcasses that became more stringent in 2014 [11] and a visual-only inspection of slaughtered pigs that are considered to pose low risk [12]. Exemptions of *Trichinella* testing of pigs originating from farms with high biosecurity level as well as when their meat undergoes specific freezing treatment are regulated [13]. Also, process hygiene criteria for *Campylobacter* in broiler carcasses have recently been introduced [14]. The new legislation on official controls [15] will ensure, among other things, the application of food law and rules on animal health and welfare; finally, revised meat inspection procedures for all species [16] apply as of 14<sup>th</sup> December 2019.

For the relatively short period of initial implementation of the new concept in practice, several challenges are seen. For instance, amendment of the “old” pig meat inspection [4] with the “new” one [12] came into force in 2014 immediately in all EU Member States (MSs); however, the practical implementation of visual-only inspection of pigs is seen only in a few MSs so far. The main reason of this postponing in most MSs is related to the insufficiency of the current FCI system that is needed to enable proper risk categorisation of farms/animals [16]. Another problem with visual-only inspection experienced so far is related to the trade agreements negotiated with countries outside the EU.

### 3. What is next?

The thorough development and fine-tuning of the new meat safety assurance system are necessary steps before its full implementation, which is expected to be relatively slow and in line with testing of the new system's feasibility in practice. To increase efficiency of regulatory controls in the modernisation process, different existing meat safety systems will have to be steadily harmonised under the general principles and framework of the meat safety assurance system to the extent achievable.

New challenges are anticipated with further evolution of the system. To enable the additional revision of relevant legislation, the European Commission will need further scientific inputs from the EFSA. Hazard identification and risk ranking will be regularly revisited and performed at regional level, which might lead to some modifications of the proposed system. Furthermore, dynamics of the new system's development process are expected to be variable across different countries and consequential to the alignment of current private food safety standards in meat industries with the new system.

Numerous knowledge gaps are still present and further scientific research needs to address them before the full practical implementation of the new system. Research on harmonisation and advancing the use of FCI in risk categorisation of incoming animals is needed to enable adequate carcass processing as well as meat inspection intensity. Among other things, this effort is needed to clarify use of multi-serological herd profiles for priority hazards, to improve various serological tests in animals for slaughter, to assess the effectiveness of harmonised epidemiological criteria in risk categorisation of farms/animals and abattoirs, and to improve the FCI forward and backward flows [17-19]. To support future *post-mortem* inspection, research should include further development of imaging technologies to detect lesions and faecal contamination [20]. Also, various options of future manual *post-mortem* meat inspection of the high risk animals (e.g. away from the slaughter-line to avoid any accompanying cross-contamination), and risk assessment of the cross-contamination due to palpations and incisions merit further investigations [5,7]. Assessment of the impact of visual-only inspection and contribution of specific meat inspection tasks to public health and animal health and welfare requires deeper studies [21]. Also, measures to compensate for any omitted/reduced palpations and incisions are proposed [22], but need to be assessed when practically tested; furthermore, new meat inspection alternatives targeting specific hazards should be developed [23]. Investigations to advance the use of risk reduction capacities of abattoirs are needed for the new meat safety assurance system. Promising meat decontamination technologies are proposed [24] and further studies should be carried out to optimise them and to practically assess their performances in abattoirs. New methodologies for assessing abattoir process hygiene and for risk categorisation of abattoirs need further examination as well as practical optimisation [25]. Identification of cost-effective monitoring programs for residues of veterinary drugs and other substances that are unwanted in meat [26] and use of responsive regulation approach in modernisation of meat controls [27] should be the themes of future investigations.

For the proper functioning of the new system, defining different roles and responsibilities is of the utmost importance. The risk managers are expected to play a pivotal role here, and they will link national regulatory authorities with meat industries by implementing efficient regulatory controls. They will choose which control options will be applied to ensure the meat safety targets are achieved and to make the overall most cost-effective contribution to public health. They will have to weigh up where the risks associated with non-compliance are highest, and thus where the most intrusive enforcement responses are required. This emphasises the need for proper training of the future risk managers as well as all the other participants in the new meat safety assurance system.

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# Precision livestock farming in the context of meat safety assurance system

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**Abstract.** The food chain continuum ‘from farm to fork’ should be managed to provide the appropriate level of consumer protection. Healthy animals are the essential precondition for obtaining a safe food supply. A science-based risk assessment includes the information about prevalence and concentration of major public health hazards of zoonotic origin (*Salmonella*, *Campylobacter*, *Listeria monocytogenes*, *Yersinia*, Verotoxin-producing *E. coli*/VTEC) and chemical contaminants (residues of veterinary drugs, dioxins, mycotoxins) in all modules along the food (meat) chain: farm-transport-slaughterhouse-meat processing-distribution-retail-consumers. The effective monitoring of biological or behavioural symptoms related to animal health and welfare can be achieved by Precision Livestock Farming (PLF). PLF emerged as a farm management approach based on information and communication technology (ICT). It can enable the early disease detection system using electronic information transfer from biosensors, in optimising animal health, production and management processes on farm. PLF can deliver added value to the integrated meat safety assurance system (MSAS) by providing real-time evidence of animal health and welfare status. This will strengthen understanding of all three major aspects of MSAS that must be monitored: biological hazards (farm-slaughterhouse continuum), animal health and welfare (Food Chain Information (FCI) quality and flow), and contaminants (prioritisation in monitoring based on FCI information).

## 1. Introduction

The food chain continuum “from farm to fork” should be managed to provide the appropriate level of consumer protection (ALOP). This can be achieved by science-based risk assessment which includes information about prevalence and concentration of major public health hazards of zoonotic origin (*Salmonella*, *Campylobacter*, *Listeria monocytogenes*, *Yersinia*, Verotoxin-producing *E. coli*/VTEC) in all modules along the food (meat) chain: farm-transport-slaughterhouse-meat processing-distribution-retail-consumers. Since hazards can enter the food chain at multiple points, the risk assessment should be carried out to provide knowledge about the most effective control measures which should be typically applied in a synergistic way to minimise the occurrence of food safety hazards in the food chain. Healthy animals are the essential precondition for obtaining a safe food supply. It is of the utmost importance to apply effective control measures on-farm with the aim to reduce the load of contamination in subsequent modules along the food chain. Recently, Precision Livestock Farming (PLF) emerged as a farm management approach based on information and communication technology (ICT) that is having a rapidly growing impact on the methods used in livestock production [1]. PLF can enable the early disease detection system by facilitating detection of clinical signs or symptoms (either biological or behavioural) that the animals exhibit. These signs can easily be picked up at a very early stage, to limit or avoid the occurrence of diseases, hence to avoid the use of antimicrobials, as well as to monitor animal welfare.

### 1.1. Precision Livestock Farming concept

PLF applies principles of control engineering using electronic information transfer, e.g. from biosensors, in optimising animal health, production and management processes on farm. PLF consists of measuring variables on the animals (good health, welfare, behavioural changes, good productive



performance, good reproductive performance) [2], modelling these data to select information, and then using these models in real time for monitoring and control purposes. PLF is based on the philosophy that fully automated, continuous monitoring of animals enables farmers to monitor the health and welfare status of their animals continuously and automatically and help them make quick and evidence-based decisions to adjust to changes in animal requirements, e.g. in-built collar sensors to detect specific behavioural parameters, sound or visual technologies in pig farms, that allow the continuous monitoring of behavioural/respiratory symptoms, nanobiosensors integrated with Internet-of-Things (IoT) technology for rapid and real-time monitoring of infections/animal diseases on farm. PLF supports intelligent management of animal health including rapid alert systems to meet the growing demand for animal proteins, while guaranteeing animal health and welfare, the future sustainability of animal farming, as well as improved food safety [3].

The main purpose is to obtain real-time, valid information regarding both (i) animal health (e.g. production diseases) and associated economic gains or losses, and (ii) food (meat) safety (e.g. zoonotic food borne pathogens – *Salmonella*, Verotoxin-producing *E. coli*/VTEC, *Campylobacter*, *Yersinia*) and associated consumer health issues affecting public health. Therefore, PLF is currently considered as a state-of-the-art engineering endeavour towards sustainability in (primary) food production improving, consequently, consumers' health through the more effective public health protection. The application of PLF allows optimal use of knowledge and information in the monitoring and control of processes on farm. In addition, such approach allows extension to the further step in the meat chain regarding defining the most effective control measures and risk mitigation strategies at the abattoir level. Therefore, PLF can be used strategically to support Food Chain Information (FCI) flow in the farm-to-chilled carcass continuum, as well as to facilitate decision-making by the risk managers, e.g. official veterinarian and/or authorised auxiliary appointed by the food business operator (FBO) in terms of the scope and type of the ante-mortem and post-mortem inspection. Overall, PLF can serve effectively in supporting a risk-based meat safety assurance system.

## 2. Main challenges in the livestock and meat sector

It is predicted the worldwide increase of animal products will be around 70% by 2050, which could present mankind with some serious problems [4], and the worldwide demand for meat and animal products is expected to increase by at least 40% in the next 15 years [5]. Many opinions given by the scientific community stated that the solution lies in stopping or reducing meat consumption [3]. However, it is not easy to stop or forbid people from eating meat and change their habits in a rapid manner, and these facts should not be neglected. Therefore, feasible solutions and alternatives to mitigate growing problems should be found. A major challenge within the next 10 years is how to enable continuous monitoring of animal health within big groups of animals [3]. Due to the increasing number of animals and the decreasing number of farmers, every farm will house more animals. In the future, a single farm (or animal city) could see 25,000 milking cows, 200,000 fattening pigs, or a few million broilers. Infections in such big groups can have disastrous economic and public health consequences [3]. This is also in relation to the prudent use of antibiotics in food animals and the pressure for antibiotic reduction, since overuse of antibiotics can lead to development of antimicrobial resistance in humans, associated with the food (meat) consumption [6]. For example, in the European Union (EU), study of *Salmonella* from humans, as well as *Salmonella* and *E. coli* isolates from fattening pigs and calves of less than one year of age, showed high proportions of isolates were resistant to ampicillin, sulphonamides and tetracyclines, while resistance to third-generation cephalosporins was uncommon; in *Campylobacter* isolated from humans, high to extremely high proportions of isolates were resistant to ciprofloxacin and tetracyclines [7]. In the EU in 2017, there were 643 strong-evidence food borne outbreaks (12.7% of total outbreaks), with 60% of them associated with food of animal origin, in particular meat and meat products (i.e. including meat from poultry, pork, bovine, sheep and other unspecified red meats and their products) [8], with more than 246,000 people being affected by food borne diseases, including the significantly increased antimicrobial resistance outbreaks from 10.4% in 2014 to 14.9% in 2017 [7].



While the reduction of antibiotic use is a primary challenge, the development of vaccines will take time, and the efficiency of applying vaccines in big herds must be monitored to improve them [3]. Having in mind all abovementioned issues it is evident that the animal health is a top priority in relation to human health.

### **3. Precision Livestock farming and meat safety assurance system**

PLF can serve effectively as a powerful management tool to support the integrated meat safety assurance system (MSAS). Namely, the European Food Safety Authority (EFSA) has recently proposed a generic framework for a modern, flexible and dynamic risk-based meat safety assurance system. Implementation of such a system is expected to be a slow and careful process across the EU and it would involve thorough development, fine-tuning and testing its practical feasibility, as well as general impacts [9].

#### *3.1. Meat safety perspective*

The safety of meat can be jeopardised by numerous biological, chemical and physical hazards. To tackle them, meat controls have traditionally been based on official meat inspection and/or on laboratory end-product testing. The system of traditional post-mortem meat inspection, comprising visual inspection, palpation and incision of carcasses and organs of slaughtered animals, was developed in the nineteenth century to deal with zoonotic diseases of importance at that time, e.g. trichinellosis, brucellosis, tuberculosis and cysticercosis/taeniasis. Since these diseases were relatively prevalent in Europe 150 years ago, this meat inspection was, indeed, originally risk-based. During recent decades, the scope of meat inspection has been substantially extended. Other public health issues have attracted attention, such as the presence of residues of veterinary medicines or other chemical substances, as well as TSE/BSE controls (Specified Risk Material), but also animal health and welfare protection, meat quality assurance, control of slaughter by-products and protection of the environment. Such a multi-purpose framework of control measures should provide valuable contributions to public health protection. However, many diseases for which meat inspection procedures were initially developed, 150 years ago, are rare today or even eradicated in many European countries. Meanwhile, other meat-borne diseases have emerged. Although the nature of the problems in meat safety have obviously significantly changed over time, the system of meat inspection has practically remained unchanged in Europe until modern times [10, 11]. The traditional meat inspection is not designed to deal with the currently most relevant meat-borne hazards such as *Salmonella*, *Campylobacter*, *Yersinia enterocolitica*, verocytotoxigenic *Escherichia coli* (VTEC) or *Toxoplasma gondii*, including associated antimicrobial resistance (AMR), nor with chemical hazards (residues of veterinary drugs, mycotoxins, dioxins, etc.), as these hazards usually do not cause clinical disease or result in macroscopic lesions in animals. On the contrary, the presence of lesions resulting from an infection months earlier will in many cases lead to condemnation of the meat/carcass, although the food safety burden is negligible [9].

#### *3.2. The integrated meat safety assurance system concept*

A novel concept of MSAS will be risk-based and will encompass the most relevant aspects in the farm-to-slaughterhouse continuum, as follows: (i) Biological hazards i.e. define clear targets for main hazards in/on carcasses and obtaining new data on biological hazards; develop the most effective control options for the main hazards, at both farm and slaughterhouse level; categorise herds/farms and slaughterhouses according to the magnitude of risk posed by biological hazards; and omit routine palpation or incision techniques in post-mortem inspection, (ii) Animal health and welfare i.e. design meat inspection, ante-and post-mortem, as a valuable tool for surveillance and monitoring of specific animal health and welfare conditions [12]; introduce only visual post-mortem inspection and compensate the potential loss of information regarding surveillance of animal disease and welfare with other approaches; improve the FCI quality and flow, (iii) Contaminants i.e. monitor chemical residues and contaminants based on risk of occurrence and using prioritisation based on FCI information,

introduce more flexible control programmes based on test results and addressing emerging hazards, introduce more integrated sampling, testing and intervention protocols for monitoring chemicals in the food chain and environmental contaminants [9].

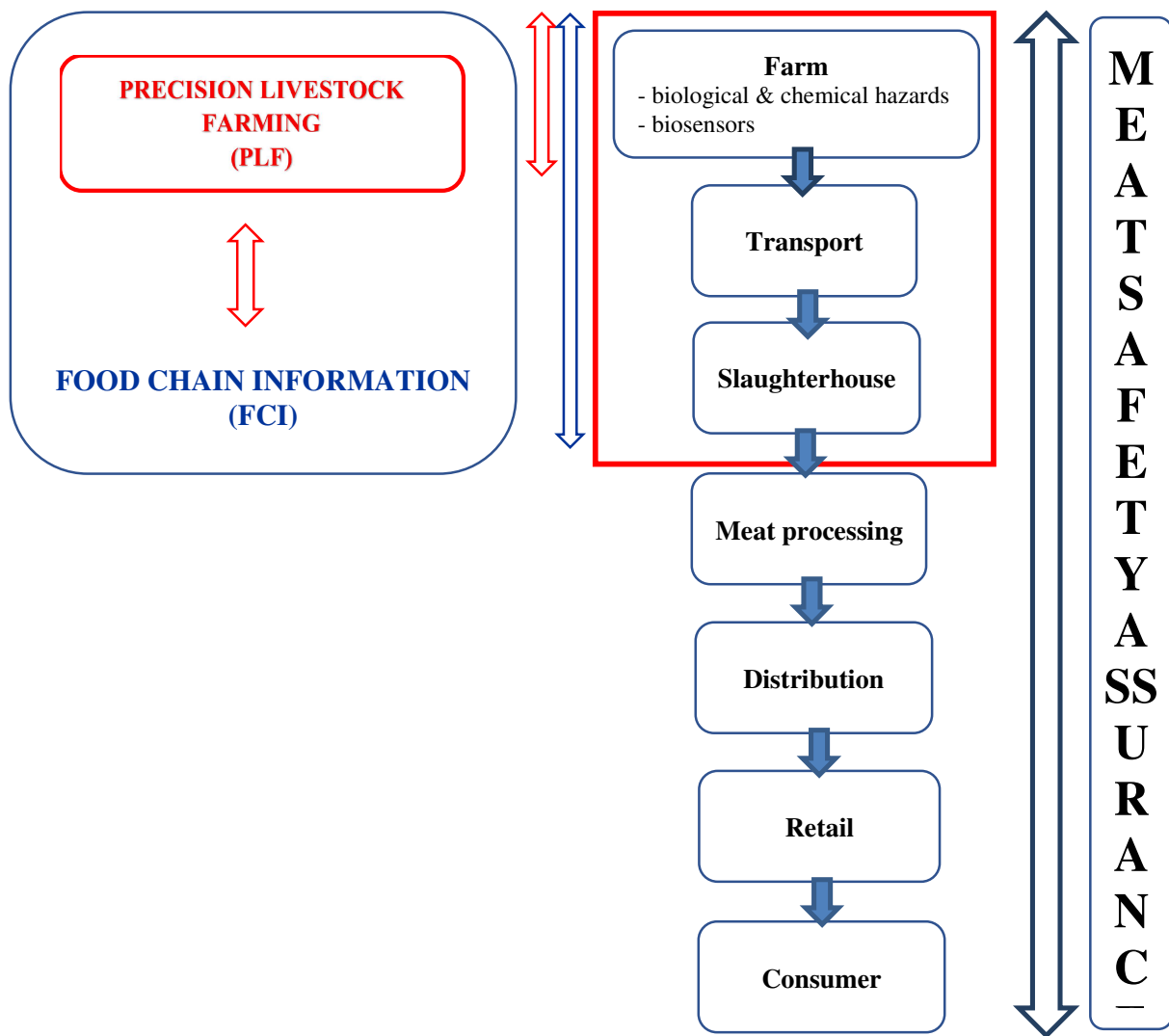
### 3.3. Interface between Precision Livestock Farming and Meat Safety Assurance System

PLF can be successfully integrated in a novel concept such as the Meat Safety Assurance System (MSAS). As a multidisciplinary approach, PLF requires collaboration among animal scientists, physiologists, veterinarians, ethologists, engineers, and information and communication technology (ICT) experts. Such an approach can serve well as support for the FCI flow and communication in the farm-slaughterhouse continuum. This, in turn, can facilitate decision-making by the risk manager (e.g. official veterinarian with assistance of authorised auxiliaries from meat business operator). Based on FCI, supplemented with valid and real-time information obtained by the PLF system, the risk manager can respond in a timely and risk-based manner, defining the scope and type of necessary meat inspection protocol in relation to the health and welfare status of incoming animals from respective farms (e.g. ante-mortem and post-mortem inspection – to omit palpation/incision or not).

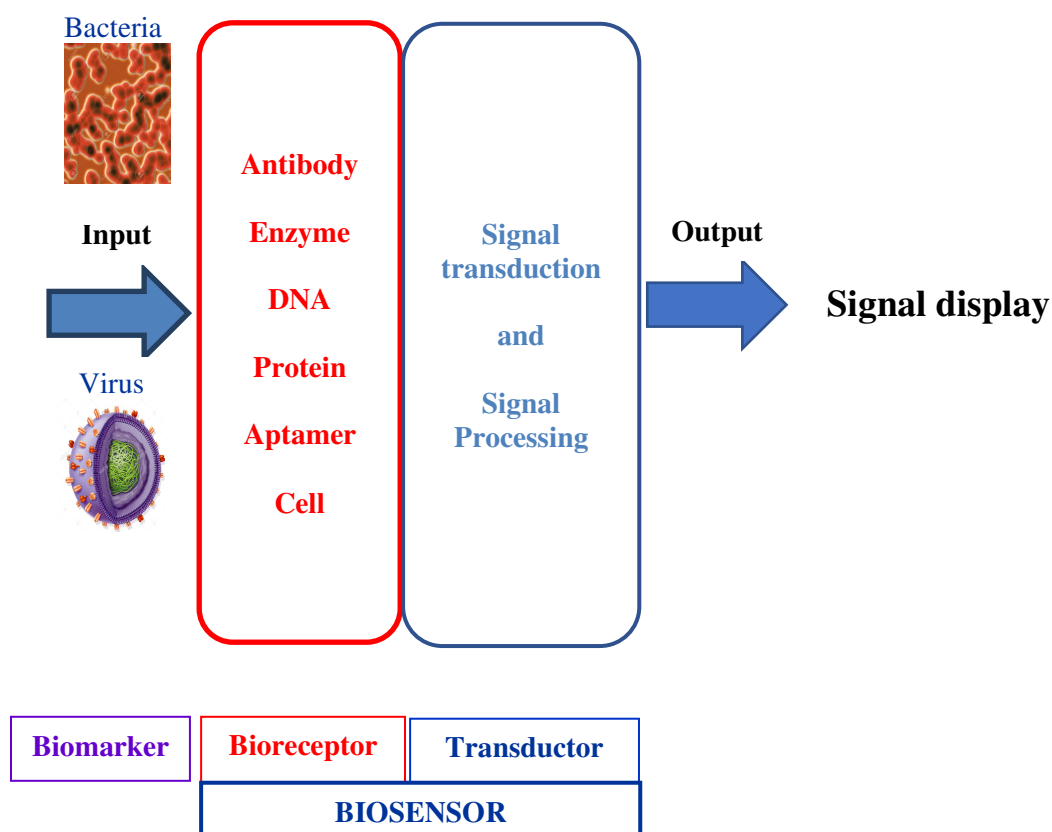
Therefore, PLF can deliver added value to the MSAS by providing automatic detection (*via* biosensors) of a variety of animal health and welfare conditions. Disturbed animal health and welfare can lead to increased faecal shedding of zoonotic food (meat) borne pathogens and consequently to increased probability for cross-contamination of animals' hides/skins/feathers on-farm/transportation/lairage, as well as carcasses at the slaughter line, and presents a food safety threat to consumers. PLF can contribute by providing real-time evidence of animal health and welfare status, which leads to better understanding of all three major aspects of MSAS: biological hazards (farm-slaughterhouse continuum), animal health and welfare (FCI quality and flow), and contaminants (prioritisation in monitoring based on FCI information) (Figure 1).

For example, the PLF approach can provide real-time (automatic) detection for the wide range of conditions related to animal health and welfare on farm [13], such as: detecting lameness in solipeds using acceleration data from ear tags, automatic 3D vision locomotion monitoring for cows, monitoring of physiological and behavioural stress in animals, monitoring of vocalisation sounds to assess response of broilers to environmental variables, pig cough monitoring as indicator of respiratory disease and environmental conditions, drinking behaviour of animals, automatic detection of health (body temperature) with a video-based infrared thermography camera, evaluating hormone profiles to improve automated oestrus detection.

From the meat safety perspective, rapid detection of major zoonotic food (meat) borne pathogens (e.g. *Salmonella*, *Campylobacter*, *Cl. perfringens*) is of the utmost importance. This can be done by the application of innovative biosensing systems (biosensors) based on specific biomarkers for pathogens affecting animal health, such as DNA receptors, glycan, aptamers and antibodies [13]. A biosensor recognises a target biomarker characteristic for a respective pathogen, *via* an immobilised sensing element – bioreceptor (monoclonal antibody, RNA, DNA, glycan, lectin, enzyme, tissue, whole cell). The specific biochemical interaction between the biomarker and the bioreceptor is then converted into a measurable signal by the transducer (Figure 2). Especially, paper-based platforms (microarrays) can be effectively used on-farm as affordable, rapid and easy to conduct sensing systems for implementation in field conditions [14].



**Figure 1.** Interface of Precision Livestock Farming (PLF) and Meat Safety Assurance System (MSAS)



**Figure 2.** Principle of biosensor based on biochemical interaction between the biomarker and the bioreceptor (adapted from [14])

#### 4. Conclusion

The food chain continuum “from farm to fork” should be managed to provide the appropriate level of consumer protection (ALOP). This can be achieved by science-based risk assessment which includes the information about prevalence and concentration of major public health hazards of zoonotic origin (*Salmonella*, *Campylobacter*, *Listeria monocytogenes*, *Yersinia*, Verotoxin-producing *E. coli*/VTEC) in all modules along the food (meat) chain: farm-transport-slaughterhouse-meat processing-distribution-retail-consumers. Healthy animals are the essential precondition for obtaining a safe food supply. PLF emerged as a farm management approach based on ICT that is having a rapidly growing impact on the methods used in livestock production. PLF can enable the early disease detection system by facilitating detection of clinical signs or symptoms (either biological or behavioural) that the animals exhibit. These signs can easily be picked up at a very early stage, to limit or avoid the occurrence of diseases, hence to avoid the use of antimicrobials, as well as to monitor animal welfare. PLF applies principles of control engineering using electronic information transfer, e.g. from biosensors, in optimising animal health, production and management processes on farm. PLF consists of measuring variables on the animals (good health, welfare, behavioural changes, good productive performance, good reproductive performance), modelling these data to select information, and then using these models in real time for monitoring and control purposes. PLF enables farmers to monitor the health and welfare status of their animals continuously and automatically and help them make quick and evidence-based decisions to adjust to changes in animal requirements. The main purpose is to obtain real-time, valid information regarding both (i) animal health (e.g. production diseases) and

associated economic gains or losses, and (ii) food (meat) safety (e.g. zoonotic food borne pathogens) and associated consumer health issues affecting public health. Therefore, PLF is currently considered as a state-of-the-art engineering endeavour towards sustainability in (primary) food production, consequently improving consumer health through the more effective public health protection it provides. Since the worldwide increase of production of animal products will be around 70% by 2050, this will present mankind with some serious problems, and the worldwide demand for meat and animal products is expected to increase by at least 40% in the next 15 years. New challenges regarding meat safety that have arisen over the previous decades are different from the classic zoonotic diseases that were relevant in the nineteenth century (trichinellosis, brucellosis, tuberculosis, cysticercosis/taeniasis); instead, emerged, current meat safety threats are associated with zoonotic meat-borne pathogens (*Salmonella*, *Campylobacter*, *Yersinia enterocolitica*, VTEC, *Toxoplasma gondii* and AMR) and chemical contaminants (residues of veterinary drugs, dioxins, mycotoxins). However, the current meat inspection system was designed more than 150 years ago and is ineffective in detecting and controlling the aforementioned emerged meat-borne threats now relevant. Therefore, there is a strong need for re-design of the meat inspection system and development of the novel concept of integrated MSAS, which will encompass all relevant modules along the meat chain, from farm to consumer. PLF can deliver added value to the MSAS by providing automatic detection (*via* biosensors) of a variety of animal health and welfare conditions. Such an approach can contribute, by providing real-time evidence of animal health and welfare status, to better understanding of all three major aspects of MSAS that should be monitored: biological hazards (farm-slaughterhouse continuum), animal health and welfare (FCI quality and flow), and contaminants (prioritisation in monitoring based on FCI information). PLF also facilitates the decision-making of risk managers (veterinary inspectors, assisted with official auxiliaries) when it comes to the scope and type of ante-mortem and post-mortem inspection at slaughterhouse level. Therefore, PLF can serve as a powerful tool for evidence-based and risk-based MSAS which should increase the public health protection, as well as reduce the economic burden to all stakeholders in the meat industry (farmers, meat business operators and consumers)

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# Wild boar meat safety

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**Abstract.** The production chain for game meat is specific and differs from the production chain of meat from domestic livestock. The aim of this study is to present the most important hazards potentially associated with wild boar meat consumption in Serbia. The most important organism is *Trichinella* spp., as the only well-established causative agent of disease outbreaks after consumption of wild boar meat. *Trichinella* spp. are endemic in Serbia and this leads to outbreaks every year. The second most important species is *Salmonella*, and there are several reports about its prevalence in the game animal population in Serbia. Chemical hazards, cadmium and lead, have also been detected in the meat and internal organs of wild boar in Serbia. These hazards are considered highly important as they reflect environment pollution, meaning they are especially important for wild boar populations inhabiting areas close to industrial plants or agricultural areas intensively treated with agrochemicals. An adequate and reliable system for the control of wild boar meat safety has to be provided. The system should be based on relevant data from our country, and thus, wild boar meat safety is an issue that requires further, comprehensive research.

## 1. Introduction

The population density of wild boars is growing in Serbia as well as in Europe, which wild boars naturally inhabit [1]. Wild boar meat is a part of the diet of the Serbian population, but in much lesser amounts than the meat of domestic animals. When assessing biohazards that potentially pose substantial risk for consumers of game meat, the fact that wild boar meat is often added to traditional meat products – fermented sausages that are not subjected to any heat treatment – must be taken into consideration [2].

The analysis of hazards relevant for wild boar meat is significantly influenced by the lack of continuous monitoring of the health status of this game animal (the monitoring system is applied only to farmed domestic pigs). Thus, diseases which are under control in primary production of domestic pigs still pose a risk for consumers of game meat. These diseases include food borne zoonoses such as tuberculosis, brucellosis, leptospirosis, etc. Besides the health status surveillance policy, another difference in the production of wild boar and domestic pig meat is the method of killing animals. Domestic pigs are slaughtered at registered facilities under controlled conditions, ensuring relevant safety of the meat. The techniques applied in hunting and carcass processing in the hunting grounds are not always in line with hygienic procedures. Consequently, there is a high rate of carcass contamination that is associated with consequent increased risk for meat contamination with intestinal pathogens [3].

Adequate risk assessment requires reliable data on wild boar habitats. In Vojvodina, which is a flatland region with extensive agricultural production, wild boars have easy access to human



settlements, domestic animals and improperly disposed animal wastes [2]. Wild animals are bioindicators of environmental pollution. Woods are specific ecosystems where chemical hazards circulate between soil, plants and wild animals, and this can lead to substantial contamination of game meat [4]. The presence of wild animals in the vicinity of industrial plants is associated with increased risk of exposure to diverse chemical hazards (e.g., PCBs). Also, the application of agrochemicals affects the contamination of game meat [5]. It is well established that after the Chernobyl accident, wild boars with increased radioactivity levels were detected across Europe [4].

The main objective of this article is to give an overview of the relevant hazards associated with wild boar meat in Serbia. The data were obtained from the official web-sites of authorized institutions, the Ministry of Agriculture and Environmental Protection and the Veterinary Directorate, for the period 2005-2016, and from relevant scientific literature.

## 2. Hazards in wild boar meat

### 2.1. *Trichinella*

Wild boar meat has been identified as a source of human trichinellosis [6]. The large numbers of diseased patients, epidemic outbreaks occurring each year, lethal outcomes, severe course of the disease, wide distribution of the pathogen across the entire country, and substantial economic losses make *Trichinella* spp. one of the most important meat borne pathogens in Serbia [7]. In assessment of game meat safety, *Trichinella* can be considered the first and most important hazard because of its high prevalence within the wild boar population and well established epidemics in humans.

Trichinellosis is a mandatory reportable disease for humans and all animal species in Serbia. According to official data from the Veterinary Directorate, during the monitored period (2005 to 2016), 2307 trichinellosis foci were reported, and a total of 3084 infected animals [8]. According to the literature data, the prevalence of trichinellosis in domestic pigs in Serbia is around 0.02%, but it can reach approximately 0.5% in endemic regions and about 1% in wild boars [9,10].

**Table 1.** Reported cases of *Trichinella* spp. in wild animals in districts in Serbia [8]

District	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	Total
Beograd			1										1
Braničevo	1			1	2	6		3	7	4	4	8	36
Jablanica					1			1	2				4
South Banat	4							3			1	1	9
South Bačka	2							1	1				4
Kolubara		1	1					1	1			1	5
Mačva									7				7
Moravica					1								1
Pirot	3	6	7	4	4	3	1	6	2	3	4	5	48
Podunavlje		1				1							2
Pčinj							1		1			2	4
Rasina							1						1
Raška				1									1
North Banat							1		1	1			3
North Bačka				2									2
Central Banat	1	1							1				3
Srem	1		1	6	8	3	2	13	13	1	5	2	55
Toplica	2												2
Zaječar	1						1	2		1			5
Zlatibor	1	3	5	3	3	2	1	2	4	4	3	2	33
West Bačka			2		1		1		1			1	6
Total	16	12	17	17	20	15	9	32	41	14	17	22	232



In the past 12 years, the presence of *Trichinella* spp. in wild animals was reported in all districts except Pomoravlje (Table 1). However, in this district, *Trichinella* spp. were reported in domestic pigs. The average number of reported game trichinellosis cases per year is 19.3 (standard deviation=8.9, min=9, max=41). The highest prevalences in wild animals (74.1%) were reported in four districts – Srem (23.7%), Pirot (20.7%), Braničevo (15.5%) and Zlatibor (14.2%). The hypothesis that there are significant differences in the prevalence of *Trichinella* spp. in wild animals between the districts is still questionable, having in mind that the presence of trichinellosis in humans and domestic livestock has been reported in the entire territory of Serbia. Instead, the differences are most likely related to the number of hunted, examined and reported wild boars (and considering that a number of cases still remain unreported).

## 2.2. *Salmonella*

According to the conclusions of the EFSA report, *Salmonella* spp. were identified as the major hazard associated with wild boar farming [11]. In Serbia, *Salmonella* is the second most important pathogen in wild boars (after *Trichinella*) because of inadequate hygienic practice during carcass processing at the hunting grounds: evisceration in the laying position on the ground and the practice of skin and interior carcass surface washing after evisceration were found to have the most significant influence on the microbiological conditions of final carcasses [3].

Our research conducted in the period 2011-2013 in Vojvodina region [3] revealed high contamination rates of wild boar carcasses. The mean aerobic colony counts (ACCs) and *Enterobacteriaceae* counts on the skin were 5.2 and 3.6 log<sub>10</sub> cfu/cm<sup>2</sup>, with 1.4% of animals' skin testing positive for *Salmonella* spp. Slightly higher mean ACC and *Enterobacteriaceae* counts of 5.4 and 3.8 log<sub>10</sub> cfu/cm<sup>2</sup> were obtained from carcass meat, with *Salmonella* spp. prevalence of 1.9%. The scant available results on the reported cases of *Salmonella* in game meat animals in Serbia are presented in Table 2 [8].

**Table 2.** Reported cases of *Salmonella* spp. in wild animals in Serbia [8]

District	2005	2013
South Banat	8	1
South Bačka	1	
Kolubara	18	
Moravica		1
Pomoravlje	2	
Rasina	1	
Central Banat	1	
West Bačka	1	
Total	32	2

## 2.3. Heavy metals

The concentrations of heavy metals can be higher in game meat than in domestic animal meat. High levels of Cd and Pb can be found in the meat of wild boars (unpublished data). Heavy metals including Cd and Pb are normally found in the soil; however, continuous industrial activities increase natural concentrations of these metals in the environment and they can be found in, beside soil, plants and animals. Contamination of plants occurs either via external contamination (dust) or absorption from the soil. Pb and Cd are two highly toxic elements with no biological function that can lead to acute or chronic poisoning of both humans and animals [12]. Cd and Pb accumulate in tissues and are more frequently found in older animals, indicating chronic exposure. Pb and Cd accumulate in the kidneys and to a lesser extent in the liver, whereas Pb also accumulates in the bones [13]. The contamination of wild boar meat is strongly associated with the animals' omnivorous diet (acorns, beech, nuts, herbs,

grass, roots, rhizomes, earthworms, insects, frogs, eggs, chicks, rodents) [14]. The uptake of Cd and Pb in wild boars occurs *via* their food or inhalation of contaminated dust. Earthworms accumulate considerable amounts of Pb as well as other heavy metals in their tissues [15]. Pigs dig up food with their snout and, thereby, consume small amounts of the soil, which can be a reservoir of contamination [16]. Pb contamination of game meat has often been attributed to the bullet; however, lead bullets are not used for hunting anymore, so this contamination source should, nowadays, not be considered. Moreover, bullets as a source of Pb contamination could be associated with the presence of lead in the region around the wound but not in the kidneys or liver. In our country, wild boars are considered important indicators of environment pollution with heavy metals. A similar situation is reported in Croatia, where Pb contents in wild boars range between 0.27-0.64 mg/kg [17]. Lower levels of Pb and Cd in wild boars were confirmed in agricultural regions with less intensive industrial activity [16, 18].

#### 2.4. Potential hazards associated with wild boar meat, for which there are no relevant data in Serbia

##### 2.4.1. Tuberculosis

Wild boars are frequently considered a spillover or dead end host rather than a true reservoir of tuberculosis. However, research conducted in Spain identified wild boars as an infection reservoir based upon the (i) presence of common genotypes in wild boar, domestic and wild animals and humans (ii) presence of tuberculosis in wild boars in fenced hunting grounds where contact with domestic livestock is absent (iii) presence of TB lesions in the lungs and potential consequent excretion, (iv) occurrence of tuberculous lesions in more than one anatomical region in juvenile wild boars [19]. So far, no data about tuberculosis in wild boars have been published in Serbia, with the exception of one report on *M. bovis* identification in one wild animal in Nišava District [8].

##### 2.4.2. *Toxoplasma gondii*

*Toxoplasma* belongs to the group of pathogens of increasing concern for food safety considering its transmission route *via* consumption of undercooked or inadequately heat-treated meat. The seroprevalence to toxoplasmosis among the wild boar population in Europe ranges around 40% [20].

##### 2.4.3. *Echinococcus*

The basic criteria for categorizing *Echinococcus granulosus* as an important foodborne parasite in Serbia are the severity of human disease and the case fatality rate. The mortality rate (number of deaths per 100,000 population) is not high, ranging from 0.01 to 0.05; however, the case fatality rate (number of deaths related to number of sick persons with the disease) is extremely high, ranging from 3.13 to 5.88% in hepatic echinococcosis, over 25.00% in *Echinococcosis alia non specificata* and as much as 33.33% in pulmonary echinococcosis. According to these data, pulmonary echinococcosis in Serbia has a fatal outcome in one third of the diseased patients [7]. Serbia, as well as other parts of the Balkans and the Mediterranean basin, is considered an endemic region for echinococcosis. According to Debeljak [5], the prevalence of echinococcosis in Serbia ranges from 2.41 to 34.8% in domestic pigs. Two cases were reported in wild boars by the same author [5]. The prevalence rates reported for pigs in some EU countries range from 1.7% in Italy to 13.5% in Spain. In Serbia, *E. canadensis* genotype G7 was reported in wild boars from Zlatibor and Kraljevo districts [21].

##### 2.4.4. Brucellosis

Porcine brucellosis in Europe is mainly caused by *Brucella suis* biovar 2, which has been considered a re-emerging disease in domestic pigs in Europe caused by spillover from the wild [22]. There are no official reports on brucellosis in wild animals in Serbia; however, the disease has been recorded in feral pigs and its presence in the population of wild boars that are in contact with such animals is highly probable [23]. Alimentary transmission of *Brucella suis* from wild boars occurs only *via* consumption of raw meat. Though the disease is of minor importance, in view of the safety of wild

boar meat, it is still considered a significant zoonosis, transmissible *via* direct contact with wild boars, aborted fetuses, etc.

#### 2.4.5. *Alaria alata*

The importance of *Alaria alata* is somewhat controversial, considering that the Federal Office for the Environment of Switzerland categorized *A. alata* as a parasite of risk grade 2 with zoonotic potential [24], but according to the most recent prioritization of food borne parasites, *A. alata* was not classified as a food borne parasite of importance [25]. *A. alata* is considered a potential cause of human disease associated with consumption of undercooked meat of intermedial hosts including game. Since wild boar meat is used in Serbia in production of traditional, non-thermally treated meat products, this risk should be considered. In Serbia, the presence of *A. alata* mesocercariae has been confirmed in wild boars from 57.1% of investigated hunting grounds in Vojvodina, with the prevalence rates ranging from 4.5 to 35.1% [26, 27].

### 3. Conclusions

*Trichinella* spp. is ranked as the top-most important hazard for human health associated with wild boars in Serbia, considering the number of reported cases of human infections after consumption of wild boar meat. None of the hazards considered here could be formally associated with this meat type. However, it is well established that a range of alimentary diseases remain unreported either due to rather mild symptoms occurring among a limited population or misdiagnosis as stomach flu (viral gastroenteritis). Chemical hazards (Pb and Cd) are of vital importance for wild boar meat safety, and further research is required to identify contamination sources and endangered localities. Putting some of the aforementioned hazards under control is feasible by regular examination of the meat after hunting (*Trichinella*, tuberculosis, echinococcosis) whereas this is not possible for pathogens such as *Salmonella*, *Toxoplasma*, or chemical hazards. Poor hygiene practices in hunting grounds substantially increase the risk that meat is contaminated with intestinal and other pathogens. Therefore, further research is of vital importance for accurate assessment of the safety of wild boar meat in Serbia.

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## Trends in chemometrics and meat products

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**Abstract.** Chemometrics is a set of mathematical and statistical methods that are used to detect food fraud, predict microbial growth, and optimize design of experiments, while extracting useful information from large and complex datasets. Complex datasets quite often have numerous sources of variations, with one or more dependent variables assessed against the two or more dependent variables, hence the need to employ some type of multivariate statistics. It is critical to decrease the chances of type I error, by comparing (calculating) all the effects of independent variables in a single multivariate test. The most common types of multivariate tests include multivariate analysis of variance (MANOVA), various forms of factor analysis (such as principal component analysis, PCA), and mathematical modeling. Bioactive compounds of plant origin possess desirable health benefits and hence are interesting for functional meat processing. The extraction and processing of bioactive compounds mostly revolve around the central problems of thermal (in)stability and environmental issues that are relevant for industry. Here, multivariate statistics can offer the best mathematical solutions for optimal industrial production or can devise various indexes that are able to follow changes of the entire chemical footprint during the extraction of target compounds. For instance, multivariate statistics is useful to determine optimal extraction parameters for antioxidants, while simultaneously evaluating the effects and interactions of extraction parameters.

### 1. Chemometrics in food science

Meat is one of the main sources of proteins, and its global production and processing will increase in the future [1]. As any food product on the market, processed meats must have required organoleptic, physio-chemical and food safety characteristics.

Significant developments in (instrumental) laboratory equipment have converted bench sciences like chemistry into great data generators while providing food science professionals with large amounts of data and a new problem, i.e., how to make sense of all that data [2]. As a solution, data analysts have



resorted to statistical concepts and tests cumulatively known as multivariate statistics that employ simultaneous analysis of one or more dependent variable(s) against two or more independent variables [3]. The key point here is “simultaneous comparison”, which if not used, has a tendency to inflate type I errors, meaning the data tend to falsely show effects and significances that do not really exist [4].

Dependent and independent variables can be categorized as qualitative or quantitative [5], whereby the former includes nominal (e.g. four types of meats; three types of meat seasoning); dichotomous (e.g. authentic or adulterated meat product; male or female animal), and; ordinal variables (e.g. data with some ordering, like the levels of a hedonistic scale for sensory evaluation of prosciutto). On the other hand, the latter, quantitative variables include scales, intervals, and ratios [6]. For example, in meat science, scales include length of processing in minutes, temperature of curing ham, added quantities of salt in brine etc. Intervals and ratios correspond to terms commonly used in everyday life.

Chemometrics encompasses the use of multivariate statistics and data mining to obtain valid conclusions from large data sets [7]. Multivariate tests include but are not limited to multivariate analysis of variance, numerous factor analyses (e.g. principal components analysis; PCA), mathematical modeling, and discriminant analysis [8].

Recently, chemometrics has been applied by government agencies and food industries to tackle the problem of food fraud and public concerns about the safety and quality of foods [9], to determine optimal processing parameters for various conditions and raw materials [7, 10], and to solve other problems in food science. This review aims to give a brief overview of the use of chemometrics in meat processing, focusing on mathematical modeling and PCA as the most commonly used multivariate tools in food science.

## **2. Mathematical modeling of food processing and food safety**

Currently there is a demand for fresh and natural products that are safe for consumption [11] but have the least possible amounts of synthetic additives [12]. This has led to the synthetics being replaced with natural alternatives that are usually extracted from plants (fruits, vegetables, agri-food waste, medicinal plants etc.), along with the development and application of processing techniques able to reach and retain desired levels of microbial load. With savvy food engineering, such products can also be labeled as “functional foods” and placed in the largest growing food marketing segment worldwide. Either way, there is an objective need for optimizing extraction procedures for bioactive compounds and predicting the microbial content of food products, and these needs are an important segment of food manufacturing [13]. One efficient way to achieve such goals is to use mathematical modeling.

Mathematical modeling is a common chemometric (multivariate) approach used in food science and technology to decrease experimental costs, predict outcomes, and optimize and describe complex processes [14]. Usually it has two main stages, construction and testing of potential mathematical relations [7] that are preceded with experimental design to achieve an optimal number of experimental runs [15]. The main purpose of modeling is to construct equations that are able to give good estimates of complex phenomena in a simplified manner [16]. Based on the method used, the equations constructed can be categorized as fundamental (mathematical regression) or semi-fundamental (Michaelis-Menten, Arrhenius, or predictive kinetics) models [14].

Fundamental modeling is usually done with some form of regression analysis that builds mathematical relations from raw data, and often from a large number of parameters [14]. The sheer amount of data and parameters makes it difficult to find and build relations, while an additional need is for experienced data analysts with solid statistical backgrounds to achieve such tasks. On the plus side, the fundamental models obtained are tailor-made for particular situations while being accurate and flexible [16].

Semi-fundamental models are simpler but less accurate and versatile, as they assume that one or more parameters are insignificant or constant [16].

Some examples of modeling processing parameters to obtain plant extracts that can act as natural additives in meat products have been recently reviewed [12]. These mixtures of various biologically active compounds need to contain the highest possible amounts of antioxidants [17], but thermal

degradation of the compounds during extraction should be avoided [18]. To optimally achieve that, the extraction technology employed [19] must be optimized by mathematical modeling [20]. Details comparing the advantages and disadvantages of processing technologies were reported elsewhere [21]. The example of microbial growth in meat was recently reviewed [22], and methods for managing microbial spoilage in the meat industry were previously reported [23].

### 3. PCA and meat adulterations

Aside from food safety and quality, consumers require authentic food products. Food adulteration is a very complex and worldwide problem, causing scandals and economic losses [24, 25] and even having severe repercussions for human health [26]. For instance, meat products are particularly interesting for fraudulent activities, for example, to the extent that corruption was shown in Brazil's government food safety system, when meat contaminated with *Salmonella* was served in Brazilian public schools [7]. Government, law enforcement agencies, media, and other professionals should work together to suppress food adulteration; however, all of them rely on specific information to be able to successfully do their jobs and implement the law [9, 27-29]. Here is yet another application of chemometrics, i.e. the very useful analysis of biological and chemical markers able to form a fingerprint for a particular attribute of a food. The fingerprint could, for example, define place of origin, batch, foreign components, etc. [30]. The concept behind detecting food fraud relies on comparing the fingerprint of an authentic food with that of a suspicious counterpart [31]. Factor analysis is a data tool that can be used in detecting food adulteration of meat products and their compliance with legal requirements [32-34].

PCA is one of many factor analysis methods, with the main purpose in food science of finding underlying relations among biological and chemical markers from large datasets [35]. During the process of PCA, it is important to define only those variables that are useful for detecting adulteration in the food, or to reduce the dataset to use only statistically relevant variables for forming the adulteration fingerprint (index). Hence, this procedure is also called reduction of dimensions. Some examples of using PCA to detect adulterated meats include minced lamb with duck meat [36, 37], wild deer with domestic goat meat [38], and beef with horse meat [39].

### 4. Conclusions

In conclusion, current trends show that chemometrics has various versatile applications in the analysis of large datasets that are provided by powerful, new laboratory techniques. Among the most interesting applications are optimizing the methods of processing bioactive food additives, predicting microbial growth with the aim of improving food safety and detecting/preventing adulteration of foods by analyzing biological and chemical biomarkers.

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## Innovative technologies for fruit extracts: Value-added opportunities in the meat industry

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**Abstract.** Consumers have concerns about the safety of synthetic antioxidants, and therefore, the use of natural antioxidants is increasing. Fruits are rich sources of various antioxidants that can be used in the meat industry as replacements for synthetic antioxidants. The naturally occurring antioxidants in fruit (e.g. polyphenols, carotenoids, vitamins) have attracted interest due to their bioactivity, to which many beneficial healthy effects are prescribed. It is well known that oxidation decreases the sensory and nutritive value of food products, whereas antioxidants added to foods can preserve the lipid components from quality deterioration. Therefore, the use of naturally extracted antioxidants from fruit could be useful to meet industry and consumers' expectations of safe and high-quality products. Recently, innovative extraction methods have been developed in order to obtain highly valued extracts for further industrial use. In particular, non-thermal technologies showed many advantages over traditional conventional methods, and therefore, much attention is paid to optimizing these lower temperature processing parameters to obtain higher yields and higher quality extracts. Incorporation of fruit extracts consisting of various bioactive compounds in processed meat will result in value-added products with associated health benefits.

### 1. Potential of fruit as natural antioxidants in the meat industry

In recent years, a great deal of attention has been directed towards natural and safe food products that may offer multiple health benefits. Moreover, consumers' considerable negative attention on the widely used synthetic antioxidants suggests that it is important to identify natural (functional) antioxidants to use in meat products [1].



Chemically synthesized antioxidant additives, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and gallates are usually added to food as antioxidants to prevent lipid peroxidation [2]. However, use of these antioxidants may lead to harmful side-effects, for example radiosensitization, increased toxicity of other chemicals, increased mutagen activity and increased risk of cancers [3, 4]. Therefore, alternative, plant-based antioxidants could have better biological and health effects in comparison to synthetic antioxidants, as the natural products exhibit better compatibility with human physiology [5].

Recent research has shown that fruits contain a wide variety of antioxidants (e.g. vitamins, polyphenols, carotenoids, chlorophylls) known for their health-promoting effects and nutritional values [6, 7]. To name a few sources, antioxidants can be extracted from plum (puree), prunes (dried plum), grape (skin, seeds, peel and pomace), berries (cakes and powder extracts), pomegranate (rind powder and juice), and most of the citrus fruits. Furthermore, agri-food waste and by-products provide a valuable source of various antioxidants. For instance, grape by-product extracts were effectively applied in the slowing down of meat discoloration and lipid oxidation [8]. When added to meat products, these molecules serve as a natural antioxidants and antimicrobial additives with the purpose of extending the quality and stability of foods [9].

The inhibitory effects of fruit extracts on meat oxidation are mainly attributed to the numerous bioactive compounds with *in vitro* and *in vivo* antioxidant activity. Correspondingly, a recent study highlighted the use of berry extracts, including bearberry (*Arctostaphylos* sp.), blueberry (*Vaccinium* sp.), blackberry (*Rubus* sp.), blackcurrant (*Ribes nigrum*), cranberry (*Vaccinium* sp.), cloudberry (*Rubus chamaemorus*), strawberry (*Fragaria ananassa*), and grape berries (*Vitis* sp.) for replacing/decreasing synthetic antioxidants in meat products [10]. These fruits are widely considered good sources of bioactive compounds, namely polyphenols (i.e., phenolic acids, flavonols, anthocyanins, tannins) and ascorbic acid that can act as strong antioxidants, able to decrease the damage caused by oxidative stress [11].

Additional to fruit extracts, fruit juices have also found their applications in the area of meat processing. In this regard, the addition of cornelian cherry juice as a functional additive in the production of beef burgers was found to effectively reduce lipid oxidation, and also allowed the maintenance of good sensory characteristics [12].

## 2. Innovative technologies for extraction of fruit antioxidants

Since liquid or dried fruit extracts are the most commonly used forms of natural antioxidants for incorporation in meat products, it is necessary to find the optimal extraction technique with respect to fruit type and targeted antioxidants [7]. The most frequently used techniques for obtaining extracts rich in antioxidants include conventional methods, due to their simplicity and wide range of applicability.

In this regard, some of the most common extraction methods from fruits using different solvent systems are based on Soxhlet, maceration, and hydrodistillation technologies. The effects of various extraction parameters in any type of extraction must be studied in order to achieve the highest yields and the highest quality extracts. In particular, the most important parameters for conventional extractions are referred to as being the solvent type, the polarity of target compounds, particle size, solid-to-solvent ratios, and extraction time [13]. However, the key disadvantages with conventional extraction techniques (compared with novel techniques) are reflected in the higher costs and environmental burden due to the use of large amounts of organic solvents, high energy consumption, extended extraction time, and higher extraction temperature, i.e., all those factors that can lead to degradation of heat-sensitive antioxidants [14].

Alternative approaches to thermal processing of food started to gain importance, above all due to increased consumer demands for new methods of food manufacturing that retain the original nutritional content and overall quality of the food. Therefore, numerous new alternatives, including high pressure, ultrasound, microwave, supercritical fluid, electrotechnologies (e.g. cold plasma, pulsed

electric field, and high voltage electric discharge) have been developed in order to overcome the main drawbacks of conventional thermal techniques [15].

The basic principles of these innovative technologies for the extraction of bioactive compounds from fruits are given in Table 1. The beneficial aspects of these approaches are the elimination or reduction of organic solvents, decreased extraction time, lower operating temperatures (preventing thermal degradation), lower energy cost, high quality extracts, improved extraction yields and high product quality and purity, hence indicating their potential for energy-efficient and environmentally friendly food processing [14, 16-18]. Regardless of the extraction technique, all methods should be optimized for the best results in the economic and nutritional senses [19].

Several innovative technologies have already been applied to various industrial sectors, where the use of water and alcohol, which are well-known versatile and eco-friendly solvents, is on the rise. In particular, Pressurized Hot Water Extraction (PHWE) is gaining more attention and is recognized as the most favored environmentally friendly technology (green technology) that operates above the atmospheric boiling point of water (100°C/273 K, 0.1 MPa), but below the critical point of water (374°C/647 K, 22.1 MPa). Under these conditions, water largely changes its chemical and physical properties, which allows the dissolution of less polar compounds from fruit matrices. Hence, this technology provides the opportunity for extraction of antioxidants with various polarities that can synergistically act as additives in meat products.

**Table 1.** Innovative technologies for the extraction of bioactive compounds in fruits

Article I. technique	Extraction	Article II. Basic principles	Article III.	References
High Pressure (HHP)	Hydrostatic Pressure	Operates at high pressures (100-1000 MPa) at 0 °C to less than 100°C for a short period.		[20-25]
Ultrasound assisted (UAE)		Ultrasound is a cyclic pressure wave that has a frequency of 20 kHz-10 MHz. Rapid changes of pressure in a liquid lead to the formation of small vapor-filled cavities – a phenomenon called cavitation. The production, growth, and collapse of the bubbles to form pores facilitate the extraction from the plant matrix.		[18, 26-31]
Microwave assisted (MAE)		Microwaves are electromagnetic radiation in the range of 300 MHz to 300 GHz. This method uses microwave energy to heat the solvent and facilitates the relocation of target compounds to the solvent. Two transport phenomena occur at the same time, namely heat and mass gradients.		[32-35]
Supercritical fluid (SFE)		Supercritical fluid extraction is characterized by changes in temperature and pressure (above its critical values) that transform a gas into a supercritical fluid.		[36-39]
Pressurized liquid (PLE)		The extraction occurs at elevated pressures (~10 MPa) and the solvent may remain in liquid state even when used at		[40-44]

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	temperatures above their boiling points	
Pulsed electric field (PEF)	Method uses short pulses of electricity ( $\mu$ s to ms) under high intensity electric fields (0.1-20 kV/cm), number of pulses <100, which leads to the formation of pores (temporary or permanent) on the cell membranes, thereby improving the extraction and diffusion processes (a phenomenon called electroporation).	[23, 31, 45-47]
High voltage electrical discharges (HVED)	Electrotechnology that damages the cell structure and promotes the extraction of valuable cellular compounds, based on the phenomenon of electrical breakdown in water. Operates at electric pulse 40 kV/10 kA and number of pulses >100.	[48-52]

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### 3. Conclusions

In conclusion, addition of mixed (synergetic) polar/nonpolar antioxidants extracted from fruits to processed meat will result in less perishable but healthier products, due to decreased oxidation and associated health benefits. With some application of savvy food engineering, such products can be easily labeled as functional. These products are expected to aid in improving consumer health while providing economic benefits that follow from placing such high demand foods on the market.

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# Application of essential oil and supercritical fluid extracts in meat processing

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**Abstract.** The meat industry is demanding antioxidants from natural sources to replace synthetic antioxidants because of their potential negative health consequences. These compounds are classified as generally recognized as safe (GRAS), and their application alone or combined with essential oils, ingredients or preservation technologies have beneficial effects on meat products. Although steam distillation is the most common industrial technique for essential oil extraction, novel technologies such as supercritical fluid extraction have emerged to address the drawbacks of the traditional extraction method and to obtain high-quality essential oils. Numerous studies have demonstrated the efficacy of essential oil obtained using the traditional or a novel extraction technique as natural antioxidants and antimicrobial agents in meat products. Based on this literature review, it can be concluded that essential oil addition in fresh and processed meat and meat products can delay, retard, or prevent lipid oxidation, retard development of off-flavors (rancidity), improve microbiological quality and extend shelf-life.

## 1. Introduction

Meat and meat products are predisposed to quality deterioration due to their complex nutritional composition that consists of different saturated and unsaturated lipids, proteins, carbohydrates, vitamins and pigments [1,2]. The quality deterioration is due to chemical degradation and microbial growth. The most common reason for chemical degradation is lipid oxidation. Lipid oxidation leads to rancidity, discoloration and accumulation of potentially toxic compounds that are harmful to human health. Hence, suppressing lipid oxidation and preventing bacterial growth are major means of extending the shelf life of meat and meat products [3,4]. Beside packaging, one of the main strategies to suppress lipid oxidation and inhibit microbial growth is the application of synthetic antioxidants and antimicrobial agents [5]. However, these compounds are potentially unhealthy due to their toxic, mutagenic and carcinogenic properties [6-8].

Consequently, in the past decade, the interest of researchers has been focused on finding alternatives for these additives from natural resources [9-17]. It is well known that plant essential oils (EOs) and extracts exhibit strong antioxidative and antimicrobial activities and possess anticarcinogenic and antimutagenic properties. Hence, the addition of plant EOs and/or plant extracts could be a good solution to improve the health image of different types of meat products [6-9].

EOs are volatile, oily extracts obtained from aromatic and medicinal plant materials, including flowers, buds, roots, bark, and leaves by means of expression, fermentation, extraction or steam



distillation, and have strong antioxidant and antimicrobial activities [18]. Approximately 300 EOs are commercially important in the fragrance markets. Due to their biological properties and flavor characteristics, these oils have been extensively used for centuries in food products. Regarding meat and meat products, EOs from oregano, rosemary, thyme, clove, balm, ginger, basilica, coriander, marjoram and basil have shown great potential to be used as antioxidants and antimicrobial agents [2,18,19].

## 2. Essential oil extraction technique

EOs and extracts from different plant materials can be obtained using different extraction techniques. Hydrodistillation and supercritical fluid extraction (SFE) have been frequently used for EO recovery, while diverse emerging techniques such as microwave-assisted, ultrasound-assisted and subcritical water extraction have been used for isolation of the polyphenolic fraction [20,21,22].

Conventionally used techniques for EO recovery such as hydrodistillation and extraction with organic solvents are accompanied by certain disadvantages. High temperatures during hydrodistillation can lead to decomposition of thermo-labile compounds, which results in variation of chemical shape in the EO obtained. Moreover, the hydrodistillation technique demands enormous energy consumption. In the case of EO extraction with organic solvents, toxicity of the solvent residue and its poor selectivity are the main drawbacks. Therefore, new trends of “green” chemistry demand application of “green” extraction techniques. Additionally, it has been noticed that green extraction methods, such as SFE techniques, provided certain advantages in relation to chemical shape and selectivity comparing to conventional recovery of EO [5,20,21,22].

### 2.1. Supercritical fluid extraction

SFE is marked as an advanced and environmentally friendly alternative to conventional solid–liquid or liquid–liquid solvent extraction both for analytical sample preparation and for production-scale applications, mainly when a clean solvent, such as carbon dioxide, is used instead of toxic organic solvents. The advantages of SFE are the possibility of tuning the solvent power of the fluid by changes in pressure and temperature, while simultaneously modifying other physico-chemical properties such as density, viscosity and diffusivity. In general, transport properties are favored under supercritical conditions, and therefore, extraction processes are faster and provide higher extraction yields [20–22].

Yousefi *et al.* [22] concluded that the resulting supercritical fluid extracts are clean and pure, possess high quality, and their aromas have great similarity to the aromas of the original plants before the extraction process. The temperatures used in the extraction (around 35°C) allow this process to be used for thermally and chemically sensitive compounds, maintaining the quality of the final product.

## 3. Application of essential oils and supercritical fluid extracts in meat and meat products

In the past decade, many studies examined the application of EOs as antioxidant and antimicrobial agents in meat products. These natural antioxidants were obtained from different herbs and spices (rosemary, oregano, sage, thyme, basil, winter savory and others) and their potential for decreasing lipid oxidation and microbial growth in meat and meat products was investigated [3–9,23–31].

Rosemary EO as a natural antioxidant was used in several studies [19,23,24]. This EO had a good antioxidant potential in fresh sausages [23] and cooked pork sausages [19,24].

Oregano EO's role as a natural antioxidant was confirmed in different types of meat products. Fasseas *et al.* [25] suggested this EO was suitable for use in raw and cooked porcine and bovine ground meat. Oregano EO at concentration of 3% w/w produced a significant decrease of the oxidation reactions in analyzed meat samples. Also, Viuda-Martos *et al.* [19,26] determined that oregano EO decreased lipid oxidation and microbial growth in meat products.

Sage EO was also evaluated in other studies [3,27,28]. Estévez *et al.* [27] suggested that sage EO (0.1%) had a good antioxidative potential in liver pâté during 90 days of storage.

Thyme EO was used as a natural food preservative in different meat products. This EO effectively delays lipid oxidation in minced beef [29], chicken breast [30] and fresh pork sausages [8].

Basil EO was also used as a natural antioxidant in a meat processing [9,31]. The effect of different concentrations of basil EO (0.062, 0.125, and 0.25%) on the lipid oxidative stability of burger was assessed during 12 days of refrigerated storage at 4°C [31]. Basil EO reduced lipid oxidation of beef burger and its efficiency was not dependent upon the EO concentration. Also, in our previous study [9], we determined that basil EO at lower concentrations (0.1-1.0 µL/g) had positive effects on the oxidative and microbial stability of cooked pork sausages during 30 days of refrigerated storage.

It is well known that SFE has been utilized for extraction of EOs (producing SFE-EOs), due to its advantages over the conventional hydrodistillation technique [20-22]. Hence, in our previous studies we examined the effects of sage and winter savory EOs obtained by conventional hydrodistillation and SFE as potential antioxidants and antimicrobial agents in fresh pork sausages [4,5,28]. Conventional EO and SFE-EO obtained from sage herbal dust were added at three concentrations (0.050, 0.075 and 0.100 µL/g) to fresh pork sausage mixture (*Petrovská klobása* sausages) [4,28]. Sage SFE-EO at concentrations of 0.075 and 0.100 µL/g was the most effective against microbial growth [28]. This study demonstrated the good antioxidative potential of sage EO and especially of sage SFE-EO, which produced the greatest inhibitory potential against lipid oxidation at a concentration of 0.100 µL/g [28].

Antioxidant effects of EOs could be achieved through scavenging free radicals, the inhibition of lipid peroxidation, and the chelating of transition metal ions [1,2,28]. Besides monoterpene hydrocarbons, diterpene polyphenols were designated as the major subgroup of sage bioactive compounds responsible for high antioxidant potential. The higher antioxidant activity of sage SFE-EO could be potentially explained by the synergistic effects of terpenoids and other lipids which are simultaneously extracted by SFE [28]. Moreover, sage SFE-EO provided better sensory properties of fresh pork sausages, another advantage of this novel extraction technique [28]. Yet another aspect which should be considered is utilization of sage herbal dust as raw material for EO recovery and utilization in food products. Valorization of by-products such as this would lead towards more sustainable and more economically efficient production of natural extracts [4,28]. The overall results show that sage EO and especially sage SFE-EO, as natural antioxidant and antimicrobial agents, could be successfully applied in meat processing [28].

In the case of winter savory, EO and SFE-EO were added at concentrations of 0.075 and 0.150 µL/g in basic formulations for fresh pork sausage. Winter savory EO and SFE-EO improved the oxidative stability of fresh pork sausages. The measured good antioxidant activity of winter savory lipid extract obtained by SFE could be attributed to its major monoterpene phenolics, particularly carvacrol and thymol. Winter savory EO and SFE-EO at 0.150 µL/g both reduced the *Enterobacteriaceae* count in sausages to under 3 log cfu/g. However, sausages produced with winter savory SFE-EO achieved higher scores for odor, flavor and overall acceptance than did sausages with winter savory EO. Therefore, this study revealed the significant antioxidative and antimicrobial activity of winter savory SFE-EO, and consequently its potential for utilization in meat industry [5].

#### 4. Conclusions

Meat and meat products are highly susceptible to lipid oxidation and microbial deterioration, which ultimately lead to safety and quality issues. EOs could be used in meat and meat products as natural alternatives to synthetic food additives, particularly as effective antioxidant and antimicrobial agents. However, EOs can negatively modify the sensory properties of the final product due to their strong aromas. The novel extraction technique, SFE, should be optimized to achieve optimal composition of EOs regarding antioxidant and antimicrobial activity as well as sensory quality of meat products.

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# Antilisterial effect of juniper (*Juniperus communis*) and its mixed application with winter savory (*Satureja montana*) in beef protection

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**Abstract.** *Juniperus communis* essential oil and post-distillation waste were tested for their antibacterial potential against common food contaminants. Results of microdilution assay directed further study of *Listeria monocytogenes*. Checkerboard assay showed synergistic antilisterial effect of both substances with conventional antibiotics. By evaluating of their cytotoxicity on human colon cells, post-distillation waste was denoted as selective against *L. monocytogenes*, being able to interfere with its *in vitro* adhesion on colon cells. On the other hand, the food preservative potential of *J. communis* essential oil was studied and compared with the activity of *Satureja montana* essential oil. In a microdilution assay, both substances induced antilisterial effect against reference ATCC 19111 strain and three wild isolates. A checkerboard assay showed synergism against isolates. An *in vitro* time-kill assay was used to confirm the types of interaction, and to estimate curve MIC values. Finally, *in situ* antilisterial efficacies of the individual essential oils and their mixture on red wine-marinated beef, previously inoculated with ATCC 19111 strain or primoisolate from beef carcass, were determined. All treatments enhanced the antilisterial potential of wine marinade. In conclusion, derivatives of *J. communis* possess significant antilisterial potential both in *in vitro* and *in situ* conditions, so further research is advised.

## 1. Introduction

In recent years, interest in plant-derived food additives and preservatives has expanded for numerous reasons. Synthetic additives and preservatives can induce different side effects, including altering biochemical parameters and inducing oxidative stress [1,2], allergenic and hypersensitivity reactions [3,4], as well as inducing genotoxic and/or carcinogenic effects [5]. On the other hand, growing data confirming different health-promoting properties of natural flavors and preservatives is additionally strengthening this area [6]. Among aromatic and medicinal plants that are used in food preparations, common juniper (*Juniperus communis* L.) is important for food and beverage preparations. Juniper is a common meat seasoning, used to impart a sharp, clear flavor to meat, especially of game, wild birds and pork [7,8]. Furthermore, it is used to aromatize gin and the local juniper brandies manufactured in Slovenia, Slovakia and Serbia [9,10]. *Juniperus* species are also used in traditional medicine. Scientific studies have revealed juniper's numerous biological activities, including antioxidant and anti-inflammatory [11], nephroprotective and hepatoprotective effects [12,13], as well as adjuvant cytotoxicity against cancer cells [14]. Moreover, the antimicrobial effect of *Juniperus* plants has also been reported [15-17]. Although antimicrobial effects were partly investigated previously, the effects of juniper had not been specifically directed toward foodborne pathogenic and spoilage bacteria, and had not been investigated in *in situ* conditions. That directed our research, and we studied the selective toxicity of *J. communis* derivatives against food contaminants [18], as well as *in situ* antibacterial effects of *J. communis* essential oil (EO) applied in wine-marinated beef individually and in combination with *Satureja montana* EO [19]. The aim of this paper is to report the results of our recent studies concerning the antibacterial potential of *J. communis* derivatives.



## 2. Antibacterial effect of *J communis* derivatives and their synergistic potential to enhance conventional antibiotics activity

Juniper berries were collected from Stara Planina Mountain in July 2014. Distillation of EO was performed in Clevenger-type apparatus, while the aqueous solution remaining after distillation was used to prepare post-distillation waste (PDW), both as described by Vasiljević et al. [14]. Since juniper EO production is extensive, mainly due to the beverage, cosmetic and perfumery industries [20], the potential use of PDW as a source of biologically active substances seem to be of great importance. Chemical characterization of EO and PDW was provided by GC-MS and LC-MS/MS, respectively. Dominant constituents, amounting to more than 1% of EO and more than 0.1 mg/g of PDW, are listed in Table 1.

**Table 1.** Chemical composition of *Juniperus communis* essential oil and post-distillation waste

Essential oil compound	content (%)	Post-distillation waste compound	Content (mg/g of dw)
		Quercetin 3-O-rhamnosylglucoside (Rutin)	12.25 ± 0.37
α-Pinene	23.61	Quinic acid	11.09 ± 1.11
δ-Cadinene	10.71	Catechin	5.534 ± 0.553
Sabinene	9.53	Epicatechin	1.738 ± 0.174
Germacrene D	7.25	Amentoflavone	0.392 ± 0.012
α-Muurolene	6.58	Umbelliferone	0.253 ± 0.025
γ-Cadinene	5.87	Quercetin 3-O-glucoside	0.232 ± 0.007
Germacrene B	4.56	(Isoquercitrin)	
β-Elementene	4.37	Protocatechuic acid	0.145 ± 0.012
α-Humulene	3.08	Apigenin 7-O-glucoside	0.140 ± 0.007
		Quercetin 3-O-rhamnoside	0.139 ± 0.008
β-Caryophyllene	2.94	(Quercitrin)	
Vidrene (thujopsene)	2.66		
Limonene	2.07		
α-Cadinene	1.83		
β-Myrcene	1.36		
4-Terpineol	1.26		
γ-Elementene	1.31		
cis-Muurolo-4(14),5- diene	1.15		
β-Pinene	1.10		

The antibacterial potential of *J. communis* EO and PDW against several reference strains of common food contaminants, monitored in microdilution assay as previously explained [21], revealed that *Listeria monocytogenes* was the most sensitive, especially in the case of PDW (minimal inhibitory concentration - MIC value 0.39 mg/mL, Table 2). Since the intention was to determine the adjuvant potential of *J. communis* derivatives to enhance the activity of common antibiotics, their effectiveness was also determined in a microdilution assay.

Effects of combinations of EO/PDW with ampicillin, azithromycin and streptomycin were estimated in the checkerboard assay. Type of interaction was determined by calculating fractional inhibitory concentration index (FICI) for two antimicrobial agents applied in combination, as in Mulyaningsih et al. [22]. The results obtained pointed to the synergistic enhancement of antibiotic activity against *L. monocytogenes* and *Staphylococcus aureus* (Table 3), while the best type of interaction between co-tested compounds against *Shigella flexneri* and *Pseudomonas aeruginosa* was additivism (data not shown). PDW synergistically increased the antimicrobial potential of all three



antibiotics against *L. monocytogenes*, as well as of azithromycin against *S. aureus*, while EO induced synergistic effects with ampicillin and streptomycin. Since antibiotic resistance is considered to be a serious global public health problem, and because antibiotic use can have side effects both on human health and the environment [23-25], this result is important.

**Table 2.** Antibacterial effect of *Juniperus communis* derivatives and conventional antibiotics

Bacterial strains (ATCC)	PDW (mg/ml)		EO (mg/ml)		Amp <sup>1</sup> (µg/ml)		Azm <sup>2</sup> (µg/ml)		Str <sup>3</sup> (µg/ml)	
	MIC <sup>4</sup>	MBC <sup>5</sup>	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
<i>Salmonella Enteritidis</i>	12.5	>25	>50	>50	800	800	50	100	3.125	12.5
<i>Escherichia coli</i>	6.25	>25	>50	>50	160	>160	50	100	6.25	6.25
					0	0				
<i>Shigella flexneri</i>	3.125	25	12.5	25	50	100	6.25	12.5	1.56	1.56
<i>Pseudomonas aeruginosa</i>	3.125	>25	>50	>50	160	1600	100	>100	12.5	50
					0					
<i>Staphylococcus aureus</i>	3.125	12.5	6.25	12.5	12.5	25	1.56	25	6.25	12.5
<i>Listeria monocytogenes</i>	0.39	0.78	3.125	6.25	50	50	3.125	6.25	12.5	12.5
<i>Enterococcus faecalis</i>	12.5	25	12.5	12.5	160	1600	25	25	200	200
					0					

<sup>1</sup>Amp – ampicillin; <sup>2</sup>Azm – azithromycin; <sup>3</sup>Str – streptomycin; <sup>4</sup>MIC – minimal inhibitory concentration; <sup>5</sup>MBC – minimal bactericidal concentration

**Table 3.** Minimal FICI\* values that indicated synergistic antibacterial effects of *Juniperus communis* derivatives and conventional antibiotics

<i>L. monocytogenes</i>					<i>S. aureus</i>
PDW+amp	PDW+azm	PDW+str	EO+amp	EO+str	PDW+azm
0.281	0.281	0.156	0.187	0.312	0.250

\*FICI = (MIC<sub>EO/PDW in comb</sub>/MIC<sub>EO/PDW alone</sub>) + (MIC<sub>antibiotic in comb</sub>/MIC<sub>antibiotic alone</sub>); Type of interaction is considered synergistic if FICI ≤ 0.5

### 3. Antilisterial effect of *J. communis* derivatives – selectivity and antiadhesive potential

Accordingly to results of microdilution assay, further investigation was focused on *L. monocytogenes*, as it was the most sensitive of the examined bacteria to EO and especially PDW. Bearing in mind that *L. monocytogenes* is an invasive food-borne pathogen that enters the host mainly in the intestine [26], we considered study of *J. communis* derivatives on human intestinal cells to be important. We used *in vitro* colon cell line models and determined the cytotoxicity against human cells, which enabled us to estimate if they were able to selectively kill only bacterial cells. Cytotoxicity was monitored in the MTT assay and IC<sub>50</sub> values were determined [27]. Calculation of selectivity index (SI) values was performed as previously described [28]. The results obtained indicated that PDW induced lower cytotoxicity against both used colon cell lines, and therefore, PDW was selectively toxic to *L. monocytogenes* (Table 4). PDW, as a highly selective antilisterial agent, was further screened in *in vitro* adhesion-inhibition assay, performed as previously described [29]. This assay determined PDW's potential to inhibit adhesion of *L. monocytogenes* to human intestinal host cells. Results revealed that a sub-inhibitory concentration (1/2 MIC) of PDW notably prevented *L. monocytogenes* adhesion to intestinal cells. The inhibition of adhesion for HT-29 and HCT116 cell line was 29% and 62%, respectively.

**Table 4.** *J. communis* EO and PDW: selective toxicity against *L. monocytogenes* and reductive potential of its adhesion on human colon cell lines HCT-116 and HT-29

	<i>L. monocytogenes</i>	HCT 116			HT 29		
	MIC (mg/ml)	IC <sub>50</sub> (mg/ml)	SI <sup>1</sup>	%Iadh <sup>2</sup>	IC <sub>50</sub> (mg/ml)	SI	%Iadh
EO	3.125±1.22	0.16±0.06	-1.3	nd*	0.10±0.03	-1.6	nd
PDW	0.39±0.07	6.84±1.43	1.2	62.0±5.4	1.45±0.52	0.6	29.4±3.9

<sup>1</sup>SI (selectivity index) = log (IC<sub>50</sub>/MIC); positive SI value indicates higher toxicity against bacteria (with the increase of selectivity, value of SI is also increasing); <sup>2</sup>%Iadh (inhibition of adhesion of *L. monocytogenes* to colon cells); \*nd - not determined

#### 4. Antilisterial effect of *J. communis* and *S. montana* EOs - *in vitro* screening of primoisolate susceptibility

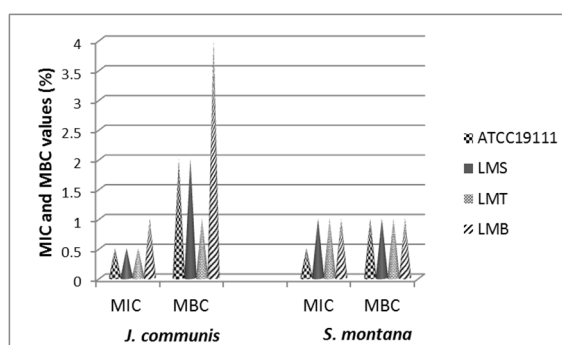
In further work, we focused on possible applications of *J. communis* EO in biocontrol of *L. monocytogenes* in meat. Taking into account that *J. communis* could be used individually or in combinations with EOs of other spices/herbs, *Satureja montana* EO was involved in this part of the research. It was selected in accordance with preliminary sensory evaluation (data not shown). In addition, the antilisterial effect of this oil could be used as reference value, since it has previously been well documented [30]. The EOs used in this part of the study were commercially provided and chemically characterized by GC-MS (Table 5), as previously described by Vasiljević et al. [19].

Comparative analysis of the EOs' antilisterial effects was performed in microdilution assay, applied to two primoisolates from foods (isolates LMB and LMS, from beef carcass and salmon, respectively), one slaughterhouse environmental isolate (isolate LMT, from water drainage tunnel), and one reference strain ATCC 19111. MIC values were determined to be 0.5-1% (Figure 1).

**Table 5.** Chemical composition\* of commercially provided *J. communis* and *S. montana* EOs

<i>Juniperus communis</i> EO		<i>Satureja montana</i> EO	
Compound	content (%)	compound	content (%)
α-Pinene	47.8	Carvacrol	30.7
Sabinene	11.0	Thymol	18.0
β-Pinene	8.5	<i>para</i> -Cymene	15.6
Limonene	5.8	Borneol	5.9
Myrcene	3.4	γ-Terpinene	5.5
<i>para</i> -Cymene	2.9	cis-Caryophyllene	3.7
α-Thujene	2.7	Linalool	3.4
<i>cis</i> -Sabinene hydrate	2.5	<i>trans</i> -Muurolo-4(14),5-diene	1.5
<i>trans</i> -Verbenol	1.6	Camphor	1.3
Caryophyllene oxide	1.6	α-Terpinene	1.3
<i>trans</i> -Pinocarvyl acetate	1.3	α-Phellandrene	1.2
α-Campholenal	1.2	Camphene	1.0
		Myrcene	1.0

\*Only dominant constituents comprising more than 1% of EO are presented.



1% concentration corresponds to 10 µl/ml EOs; i.e. to 8.58 mg/ml and 9.16 mg/ml for *J. communis* and *S. montana* EO, respectively.

**Figure 1.** MIC and MBC values of *J. communis* and *S. montana* EOs determined in microdilution assay performed on *L. monocytogenes* ATCC 19111 reference strain, and on isolates from salmon (LMS), slaughterhouse water drainage tunnel (LMT), and beef carcass (LMB).

The combined effect of the EOs was monitored in checkerboard and in time-kill assays, both applied as previously explained [19]. Screening in the checkerboard assay was performed with all tested isolates, while the time-kill assay was applied only to LMB isolate and ATCC 19111 strain. Results obtained in the checkerboard assay showed that synergism was determined for some oil combinations in the case of all isolates, while interaction of EOs active against ATCC 19111 was, at best, additive (Table 6).

**Table 6.** Synergistic and additive interaction between *J. communis* and *S. montana* oils determined by FICI values

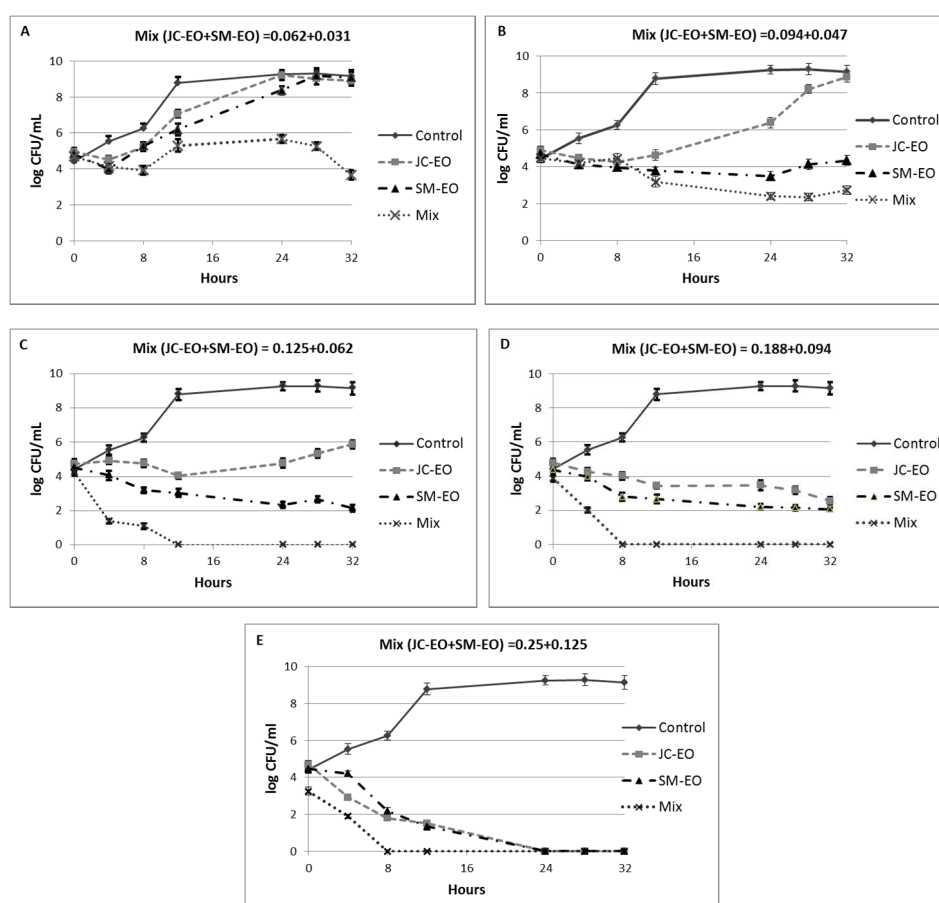
conc. <i>J. communis</i> [%] Interpretation	conc. <i>S. montana</i> [%]		FICI*
LMS			
0.25	0.125	0.625	Additive
0.125	0.125	0.375	Synergistic
0.0625	0.25	0.375	Synergistic
0.03125	0.25	0.3125	Synergistic
0.015625	0.25	0.281	Synergistic
0.0078125	0.25	0.266	Synergistic
LMT			
0.25	0.25	0.75	Additive
0.125	0.25	0.5	Additive
0.0625	0.25	0.375	Synergistic
0.03125	0.25	0.3125	Synergistic
0.015625	0.25	0.281	Synergistic
0.0078125	0.25	0.266	Synergistic
LMB			
0.5	0.015625	0.516	Additive
<b>0.25</b>	<b>0.125</b>	<b>0.375</b>	<b>Synergistic</b>
0.125	0.25	0.375	Synergistic
0.0625	0.25	0.3125	Synergistic
0.03125	0.25	0.281	Synergistic
0.015625	0.5	0.515	Additive
ATCC 19111			
<b>0.25</b>	<b>0.125</b>	<b>0.75</b>	<b>Additive</b>

\*FICI = (MIC<sub>J.c.in comb</sub>/MIC<sub>J.c.alone</sub>) + (MIC<sub>S.m.in comb</sub>/MIC<sub>S.m.alone</sub>); Type of interaction is: synergistic if FICI ≤ 0.5, additive if 0.5 < FICI ≤ 1. The combination tested in time-kill assay is bolded.

Whilst for the checkerboard assay, combinations of EOs were prepared in different ratios, for the time-kill assay, only combinations containing EOs of *J. communis* and *S. montana* in the ratio 2:1 were used, in accordance with their effectiveness against LMB and ATCC 19111 in the checkerboard assay and with preliminary sensory evaluation (data not shown). Results of the time-kill assay

confirmed the additivism observed in the case of ATCC 19111 (data not shown), and synergism detected in the case of LMB (Figure 2).

Interestingly, the time-kill assay revealed higher susceptibility of bacteria than was observed in the checkerboard and microdilution assays. This was attributed to differences in oxygen availability, which consequently induced metabolic changes. As a facultative anaerobe, *L. monocytogenes* performs aerobic respiration and possesses intensive metabolism in aerobic conditions, which leads to an increase of bacterial sensitivity to different stressors, including antimicrobials [31,32]. To quantify the observed differences in sensitivity, we further used data obtained from time-kill curves and calculated the curve MIC values (cMICs). This involved plotting the growth/inhibition rates during the first 12 hours of incubation vs. concentrations of tested substances, as previously explained [19]. Determined cMICs were approximately 0.03-0.04 for *S. montana* EO, and 0.10-0.14 for *J. communis* EO, both being remarkably lower than MICs determined in the microdilution assay.



(JC-EO) and *S. montana* (SM-EO), applied individually and in mixtures (JC-EO:SM-EO = 2:1); Confirmation of synergism was observed at 24 h of incubation if the log CFU/ml determined with mixture was lower by at least 2 logs than the log CFU/ml determined with the more active single agent, as explained in [22] (panels A,C and D).

**Figure 2.** Time dynamics of antilisterial effects against LMB isolate of essential oils of *J. communis*

### 5. Antilisterial potential of *J. communis* and *S. montana* EOs - *in situ* screening on wine-marinated beef

In further work, we monitored *in situ* antilisterial effect of *J. communis* and *S. montana* EOs on red wine-marinated beef. The EOs were tested individually and in a mixture prepared in the same ratio (*J. communis*:*S. montana* = 2:1) as in the previously explained *in vitro* time-kill assay. The preparation of wine marinades for the beef sirloin steaks and the marination process are explained in detail elsewhere [19]. Certainly, the sensory acceptability of the concentrations/combinations of the EOs used was previously confirmed (data not shown). To monitor the antilisterial effects, beef steaks were inoculated with *L. monocytogenes* (ATCC 19111 and LMB, final concentration 5 log CFU/g) prior to the marination process. Antilisterial effects on the marinated meat were determined periodically during 15 days of refrigerated storage. Analysis of results in a time dependent manner (monitoring the time dynamics for each marinade treatment) showed a bactericidal effect was obtained during the marination period (24 h), while subsequent meat storage was mainly accompanied by bacteriostatic effects (Tables 7 and 8).

Comparison of the different marinades with saline showed the antilisterial efficacy of *J. communis* EO was almost the same as that of *S. montana* EO, while the effect of the EO mixture was even higher in the case of *L. monocytogenes* ATCC 19111. Due to their antimicrobial active substances, such as thymol and carvacrol in *S. montana* EO, and pinene, limonene and sabinene in *J. communis* EO, the overall antimicrobial potential of red wine marinades was strengthened [33]. Taking into account that literature indicates the preservative potential of *S. montana* EO [30,34], our result showing the *in situ* antilisterial activities of *J. communis* and *S. montana* EOs are comparable, is of notable importance.

**Table 7.** Effect of red wine marinades containing EOs of *J. communis*, *S. montana* and their mixture on growth inhibition of *L. monocytogenes* ATCC 19111

Day	S <sup>1</sup>	W <sup>2</sup>		W+JC-EO <sup>3</sup>		W+SM-EO <sup>4</sup>		W+Mix <sup>5</sup>	
	log CFU/g	log CFU/g	F <sub>s</sub> <sup>6</sup>	log CFU/g	F <sub>s</sub>	log CFU/g	%S	log CFU/g	%S
0	5.24±0.15	3.61±0.20	68.9%	3.71±0.18	70.8%	3.55±0.21	67.7%	3.32±0.19	63.4%
1	5.44±0.19	3.82±0.15	70.2%	3.47±0.22	63.8%	3.22±0.20	59.2%	3.14±0.17	57.7%
3	5.65±0.27	3.59±0.19	63.5%	3.30±0.22	58.4%	3.49±0.28	61.8%	2.83±0.14	50.1%
5	6.09±0.22	4.19±0.21	68.8%	3.46±0.25	56.8%	3.22±0.27	52.9%	2.43±0.15	39.9%
7	6.59±0.24	4.14±0.16	62.8%	3.32±0.18	50.4%	3.10±0.16	47.0%	2.15±0.16	32.6%
9	6.77±0.25	4.25±0.25	62.8%	3.28±0.21	48.4%	3.36±0.24	49.6%	2.70±0.11	39.9%
11	6.84±0.26	4.07±0.21	59.5%	3.44±0.18	50.3%	2.98±0.18	43.6%	2.68±0.15	39.2%
13	7.11±0.33	4.73±0.18	66.5%	3.57±0.23	50.2%	3.42±0.21	48.1%	2.55±0.14	35.9%
15	7.04±0.31	4.59±0.22	65.2%	3.04±0.26	43.2%	2.80±0.24	39.8%	2.45±0.17	34.8%

<sup>1</sup>S – saline; it was prepared as sterile solution of 0.85% w/v table salt and used as negative control, in order to estimate effects of marinades

<sup>2</sup>W – wine marinade; it was prepared with dry red wine, with addition of 0.85% w/v table salt, 0.8% w/v black pepper and 0.75% w/v garlic powder; <sup>3</sup>W+JC-EO – wine marinade prepared as the base one, but additionally containing 0.25% of *J. communis* EO; <sup>4</sup>W+SM-EO – wine marinade prepared as the base one, but additionally containing 0.125% of *S. montana* EO; <sup>5</sup>W+Mix – wine marinade prepared as the base one, but additionally containing the mixture of oils, i.e. 0.25% of *J. communis* EO and 0.125% of *S. montana* EO; <sup>6</sup>F<sub>s</sub> – Fraction of log CFU/g of the referent values detected in saline; used to quantify the efficacy of each marinade

**Table 8.** Effect of red wine marinades containing *J. communis* or *S. montana* EOs and their mixture on growth inhibition of *L. monocytogenes* LMB isolate

Day	S <sup>1</sup>	W <sup>2</sup>		W+JC-EO <sup>3</sup>		W+SM-EO <sup>4</sup>		W+Mix <sup>5</sup>	
	log CFU/g	log CFU/g	F <sub>s</sub> <sup>6</sup>	log CFU/g	F <sub>s</sub>	log CFU/g	%S	log CFU/g	%S
0	5.12±0.25	3.72±0.28	72.7	3.27±0.26	63.9	3.43±0.19	67.0	3.62±0.23	70.7
1	5.87±0.27	4.21±0.23	71.7	4.13±0.18	70.4	4.10±0.18	69.8	3.92±0.19	66.8
3	6.03±0.26	4.08±0.20	67.7	3.81±0.22	63.2	4.07±0.19	67.5	3.58±0.26	59.4

5	6.82±0.35	3.96±0.27	58.1	3.77±0.22	55.3	4.09±0.21	60.0	3.74±0.23	54.8
7	7.19±0.38	4.11±0.28	57.2	3.79±0.21	52.7	4.23±0.20	58.8	3.35±0.20	46.6
9	7.26±0.32	4.08±0.26	56.2	3.80±0.21	52.3	3.48±0.20	47.9	3.29±0.27	45.3
11	7.56±0.37	4.14±0.30	54.8	3.90±0.24	52.4	3.56±0.23	47.1	2.72±0.22	36.2
13	7.43±0.40	4.25±0.23	57.2	2.97±0.21	39.8	2.72±0.20	36.6	2.64±0.19	35.5
15	7.26±0.30	4.34±0.21	59.8	2.36±0.21	32.5	3.28±0.23	45.2	2.98±0.22	41.0

All footnotes provided after Table 7 are applicable also for Table 8.

## 6. Conclusion and future perspectives

Taken together, the results obtained show the antilisterial effect of *J. communis* derivatives. They have potential to synergistically enhance the activities of other antimicrobials, such as conventional antibiotics and *S. montana* EO. Furthermore, selectivity and potential to reduce pathogen adhesion to intestinal cells, which leads to disturbance of the initial steps of host colonization, was shown for *J. communis* PDW. On the other hand, *J. communis* EO possesses preservative potential and could be used as a vehicle to control *L. monocytogenes* contamination in beef.

The data obtained indicate further investigation of the antimicrobial potential of *J. communis* could be developed in several directions. Searching for active substances in both EO and PDW, as well as elucidating their underlying mechanisms responsible for the observed effects is advised. In addition, taking into account the low cytotoxicity for human cells, determining the sensory properties and food preservative potential of PDW seems to be interesting. Finally, this work indicates investigations should be performed in other food matrices, as well as against different microbial contaminants.

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# Temperature regime and formation of carcinogenic heterocyclic aromatic amines

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**Abstract.** The effects of thermal treatment conditions on the 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) levels formed in pork samples of different matrices (pork steak vs. pork mince) were studied. Oven air temperature oscillations above the set-point temperature of 200 °C were produced using an on-off controller. Oscillations that reached 1.2 °C amplitude showed formation of one-fifth to one-tenth the PhIP levels in the pork steak and mince compared to the maximum air temperature oscillations that produced temperatures of up to 211.5 °C. The MeIQx levels in pork steaks were not significantly affected by the temperature oscillations, while they were doubled in pork mince. To reduce the levels of these heterocyclic aromatic amines, an oven with precise temperature control is significantly more important than other options during the cooking of pork.

## 1. Introduction

Domestic cooking processes are rarely studied, although they can drastically modify the quality of the heated foods, in terms of nutritional and sensory characteristics [8, 5, 11]. Despite the advantages of the cooking process, one prime concern is the formation of heterocyclic aromatic amines (HAAs). The choice of appropriate methods for thermal treatment of meat to minimise formation of HAAs is a particularly important issue. Specifically, severe cooking methods with temperatures well above 200 °C (e.g., frying, grilling, barbecuing), and/or prolonged cooking times can lead to excessive formation of HAAs [2, 6, 14].

During oven thermal treatment of meat of different sizes and muscle types, two particular aspects need to be considered: the mass transfer that drives the crust formation, and the water loss by evaporation and by protein denaturation-contraction [10].

To evaluate the performance of oven-baking appliances used in domestic cooking, their characterisation in terms of oscillations in the amplitude and frequency above the set-point temperature (i.e., compliance with and oscillations above the set-point temperature) appears to be more informative than standard air-temperature measurements [1, 4, 5, 16].

To summarise the aims of the present study, it should be kept in mind that even slight variations in oven air temperature above the set-point temperature (i.e., elevated air temperatures) or greater oscillations in the air temperature can lead to increased meat-surface desiccation and overheating, and consequently might also result in increased formation of HAAs. Based on this concept, we investigated the levels of the HAAs 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) formed in pork steaks and in minced pork thermally treated in an oven. This domestic cooking appliance (i.e., oven) was chosen because they are commonly used in homes, and there appears to be no data available regarding the HAAs that can be formed as a result of set-point temperature oscillations during the heat treatment of pork meat in different forms.





## 2. Materials and methods

### 2.1. Experimental design

Fresh pork loins of standard quality were cut into ten steaks with a thickness of approximately 5 cm. These steaks were vacuum packed, immediately frozen, and kept at -35 °C. Before the analyses, the frozen steaks intended for cooking (heating) the following day were thawed overnight at 4 °C. These thawed steaks were then cut into two similar halves, to provide smaller steaks with a thickness of 2.5 cm (mean weight, 115 ±18 g). Then, one half of each smaller steak was minced and formed into a patty-shaped sample (mean weight, 80 ±1 g; using a Petri dish). The (smaller) steaks and mince were assigned as randomised pairs for thermal treatment in an oven using 10 replicate procedures, thus eliminating any effects of meat location in the muscle.

The oven used (Gorenje+ GO978X) had a defined volume of 75 L, energy consumption at venting of 0.71 kWh, and under conventional conditions of 0.94 kWh, with a connected load of 3.4 kW, a triple glazed oven door with double heat deflector, and oven cooling. The oven had modified firmware, which allowed the setting of temperature oscillations above the set-point temperature of 200 °C, but it should be noted that the average temperature in all thermal treatments was equal (205 °C). For thermal treatment of meat, the difference between the maximum and the set-point temperature is important.

The corresponding steak and mince samples were placed in the preheated oven together and thermally treated for 40 min at 200 °C (i.e., the optimal time to prepared a 400 g pork roast). Different temperature oscillations were applied during the cooking times. The actual air temperature was measured throughout these heating procedures using a spear-pointed temperature probe (Testo 177-T4, coupled to a stainless steel class 1 probe) that was inserted into the mid-point of the oven. Each individual experiment was repeated three times.

### 2.2. Calculation of cooking loss

The cooking losses from the meat samples were expressed as percentage weight loss.

### 2.3 Determination of heterocyclic aromatic amines

After 24 h of cooling at 4±1 °C, HAA levels were determined in the roasted pork steaks and minced pork. HAAs levels were determined by the method described by Santos et al. (15), with minor modifications, as described by Polak *et al.* [12].

For liquid chromatography-mass spectrometry (LC-MS) analysis, the cooked meat samples were cleaned up using solid-phase extraction (Oasis, MCX 60 mg columns). The LC-MS analyses (Agilent 1100 system) were performed on an analytical column operated under reverse phase conditions (semi-micro TSKgel ODS-80Ts column, 5 µm, 250 mm × 2 mm i.d.; Cat. N° 18151; Tosoh Bioscience LLC, Japan) at 25 °C. The separation was performed at a flow rate of 0.3 mL/min by gradient elution with 20 mmol/L ammonium formate (Cat. N° 09739; Fluka) at pH 3.2 as solvent A, and acetonitrile (Cat. N° 1.00030; Merck) as solvent B. The injection volume was 10 µL, and the internal standard was 4,7,8-TriMeIQx. The HAAs were identified and quantified according to their retention times and the spectra from reference samples of known concentrations run under the same conditions.

### 2.4 Data analysis

The experimental data were evaluated statistically using the SAS/STAT programme. Basic statistical parameters were calculated using the MEANS procedure. The data were tested for normal distributions. The effects of sample matrix (pork steak, minced pork) on PhIP and MeIQx levels were evaluated using paired t-tests, and the main effects of temperature oscillations above the set-point temperature of 200 °C (1.2 °C to 11.5 °C) and repetitions/animal (1-3) were evaluated using the general linear model procedures. The means for the oscillation amplitudes were obtained using Duncan's procedure, and were compared at the 5% probability level.

### 3. Results and discussion

#### 3.1 Cooking loss during thermal treatment

Given that the roasting of the meats took place over a relatively long period (40 min) at high temperatures ( $\geq 200$  °C), the cooking losses were relatively high, from 45% to 52%. Indeed, these are noticeably higher than the cooking losses in a study on pork grilled at 220 °C to an internal temperature of 95 °C (35%) [12]. Similarly lower cooking losses (26%) than ours were obtained for pork cooked in a commercial oven with natural convection at the oven set-point temperature of 180 °C and a temperature of 75 °C at the thermal centre [20].

In the current study, increasing the temperature oscillations from 1.2 °C to 11.5 °C above the set-point temperature of 200 °C led to cooking losses for the pork steaks of 42% to 50%, with significance seen for the lowest cooking loss at oscillation amplitudes of 1.2 °C, 5.5 °C and 11.5 °C. For the minced pork, cooking losses were from 48% to 53%, although they were not significantly different for the different oscillation amplitudes. The pork steaks showed lower cooking losses than did the minced pork. This appears to be due to differences in the microstructures of the pork steaks and the minced pork roasted at 200 °C (the temperature in the geometrical centre of the meats was ~90 °C), with greater cooking loss (and likely also HAA precursors) occurring in the minced pork than in the pork steaks (cooking loss of 51% for mince vs. 47% for steaks).

#### 3.2 PhIP and MeIQx levels

**Table 1.** Effects of temperature oscillations on heterocyclic aromatic amine (PhIP and MeIQx;  $\mu\text{g/kg}$  thermally treated sample) levels in pork steaks and minced pork and the differences in PhIP and MeIQx levels relative to the thermally treated samples. Data are means $\pm$ standard deviation, where indicated ( $n = 120$ )

Oscillation amplitude (°C)	PhIP			MeIQx		
	Pork steak	Minced pork	$P_s$	Pork steak	Minced pork	$P_s$
1.2	3.88 $\pm$ 0.80 <sup>Fa</sup>	2.37 $\pm$ 0.25 <sup>Fb</sup>	0.062	5.02 $\pm$ 2.79 <sup>a</sup>	1.71 $\pm$ 0.99 <sup>Db</sup>	0.064
1.5	4.73 $\pm$ 0.27 <sup>Fa</sup>	3.97 $\pm$ 0.55 <sup>FEb</sup>	0.052	4.88 $\pm$ 0.87	3.65 $\pm$ 0.72 <sup>BC</sup>	0.217
1.6	7.27 $\pm$ 0.58 <sup>Ea</sup>	4.66 $\pm$ 0.61 <sup>Eb</sup>	$\leq 0.001$	8.31 $\pm$ 1.16 <sup>a</sup>	4.51 $\pm$ 1.07 <sup>BAb</sup>	0.027
1.7	7.28 $\pm$ 0.36 <sup>E</sup>	7.50 $\pm$ 0.48 <sup>D</sup>	0.569	6.93 $\pm$ 1.64 <sup>a</sup>	3.05 $\pm$ 0.72 <sup>BCDb</sup>	0.007
2.7	9.71 $\pm$ 0.24 <sup>Db</sup>	11.49 $\pm$ 0.52 <sup>Ca</sup>	0.005	5.91 $\pm$ 2.15 <sup>a</sup>	2.37 $\pm$ 1.27 <sup>CDb</sup>	0.009
4.1	11.09 $\pm$ 0.64 <sup>D</sup>	11.05 $\pm$ 0.97 <sup>C</sup>	0.950	6.49 $\pm$ 1.02 <sup>a</sup>	3.60 $\pm$ 1.53 <sup>BCb</sup>	0.021
5.5	10.86 $\pm$ 1.38 <sup>D</sup>	9.92 $\pm$ 0.87 <sup>C</sup>	0.300	5.59 $\pm$ 1.43 <sup>a</sup>	3.02 $\pm$ 1.43 <sup>BCDb</sup>	0.018
7.8	15.96 $\pm$ 2.08 <sup>Ca</sup>	11.21 $\pm$ 0.85 <sup>Cb</sup>	0.031	9.23 $\pm$ 3.74 <sup>a</sup>	5.82 $\pm$ 1.37 <sup>Ab</sup>	0.094
9.3	18.58 $\pm$ 1.02 <sup>Ba</sup>	15.61 $\pm$ 2.55 <sup>Bb</sup>	0.037	6.67 $\pm$ 3.85 <sup>a</sup>	2.91 $\pm$ 1.69 <sup>BCDb</sup>	0.041
11.5	20.78 $\pm$ 1.83 <sup>A</sup>	23.04 $\pm$ 2.28 <sup>A</sup>	0.327	7.16 $\pm$ 1.93 <sup>a</sup>	2.68 $\pm$ 1.27 <sup>BCDb</sup>	0.004
$P_P$	$\leq 0.001$	$\leq 0.001$		0.117	0.001	
Overall matrix effect	11.01 $\pm$ 5.60 <sup>a</sup>	10.08 $\pm$ 5.97 <sup>b</sup>	0.027	6.62 $\pm$ 2.42 <sup>a</sup>	3.33 $\pm$ 1.56 <sup>b</sup>	$\leq 0.001$

PhIP, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline;  $P_s$ , Statistical probability of sample matrix effect;  $P_P$ , Statistical probability of oscillation amplitude effect; Means with different superscript capital letters within columns (<sup>A-F</sup>) differ significantly ( $P \leq 0.05$ ; i.e., significance of differences between different oscillation amplitudes); Means with different superscript small letters within rows (<sup>a, b</sup>) differ significantly ( $P \leq 0.05$ ; i.e., significance of differences between pork steak and minced pork).

The samples contained PhIP at 10.55  $\mu\text{g/kg}$  and MeIQx at 4.98  $\mu\text{g/kg}$  in terms of the thermally treated samples (cooked weight), and when calculated relative to the fresh samples, in terms of the wet weights (fresh weight), these were PhIP at 5.37  $\mu\text{g/kg}$  and MeIQx at 2.55  $\mu\text{g/kg}$ . The pork steaks contained PhIP at 11.01 $\pm$ 5.60  $\mu\text{g/kg}$  cooked weight and MeIQx at 6.62 $\pm$ 2.52  $\mu\text{g/kg}$  cooked weight (Table 1), levels that were considerably higher than those reported earlier [12], i.e., PhIP at 0.77  $\mu\text{g/kg}$  cooked meat and MeIQx at 0.90  $\mu\text{g/kg}$  cooked meat for their 120-g steaks that were grilled on two-plated grill at 220 °C to an internal temperature of 70 °C. Similarly, [13] reported PhIP at 2.29  $\mu\text{g/kg}$

and MeIQx at 0.23 µg/kg in pork roast (650-680 g) that was cooked at an oven temperature of 177 °C and an internal temperature of 71 °C. In comparison to the present data [19] reported similar PhIP at 13.12 µg/kg and MeIQx at 7.59 µg/kg for pork loins slices with a thickness of 2 cm, and pan-fried at a surface temperature of 204 °C to an internal core temperature of 77 °C. For the minced pork in the present study, the HAAs were measured as PhIP at 10.08±5.97 µg/kg cooked weight and MeIQx at 3.33±1.56 µg/kg cooked weight (Table 1).

Despite the relatively large variability, there were some significant differences seen for the PhIP and MeIQx levels. Thus, the effects of the sample matrix (i.e., pork steak, pork mince) and the effects of the temperature oscillations were statistically significant ( $P \leq 0.05$ ) for both PhIP and MeIQx, as both cooked weight and fresh weight (Tables 1, 2).

The highest PhIP levels were seen in the pork steak and minced pork treated under the highest temperature oscillations (11.5 °C). PhIP levels were intermediate in the samples with oscillations of 9.3 °C, and 7.8 °C, followed by the samples with oscillations of 2.7 °C, 4.1 °C and 5.5 °C, and finally were lowest with the lowest oscillation amplitudes of 1.7 °C and less. These lower levels of PhIP for oscillation amplitudes below 1.7 °C (pork steak and mince, 2.37-7.50 µg/kg cooked weight) as compared to those for the highest oscillations of 11.5 °C (pork steak and mince, 20.78-23.04 µg/kg cooked weight) are thus due solely to the approximately 10 °C difference in the oscillation amplitudes above the set-point temperature of 200 °C.

Increasing HAAs levels are produced with increasing cooking temperatures (150-225 °C), and PhIP levels are generally higher than MeIQx levels at higher temperatures [17]. In the present study with the pork steaks, MeIQx was the most abundant HAA at temperature oscillation amplitudes below 1.6 °C (4.88-8.31 µg/kg cooked weight), although the PhIP levels increased to higher levels than MeIQx when temperature oscillation amplitudes were above 2.7 °C (9.71-20.78 µg/kg cooked weight). However, for the pork mince, PhIP was the most abundant HAA regardless of temperature oscillations (Tables 1, 2).

As a consequence of the oscillations above the set-point temperature to give a 12 °C increase, both the formation and degradation of MeIQx was expected. This was seen for in the pork steaks, where the balance of the MeIQx levels was essentially independent of the temperature oscillations ( $P=0.117$ ), and remained between 4.88 µg/kg and 9.23 µg/kg cooked weight. On the contrary, in the pork mince, the MeIQx levels depended on the temperature oscillations during the thermal treatment ( $P=0.001$ ), with the highest levels seen at the oscillation amplitude of 7.8 °C.

Kondjoyan et al. [9] emphasised the meaning of the crust, the thin area close to the surface of the meat that thickens during grilling and roasting. The HAA levels in whole meat samples depend on the gradients of temperature and water content/water activity in the thickening crust [18]. As suggested later by Kondjoyan et al. [10], crust formation at the surface of small meat pieces will differ from that in larger meat pieces due to the different thickening of the crust and the migration of the HAA precursors. On this basis, the question arises as to whether there are differences in HAA levels between pork steaks and pork mince. Generally, in comparison to pork steak, the pork mince showed lower PhIP levels, although these differences were only statistically significant under some of the temperature oscillation conditions (Table 1; 1.6, 2.7, 7.8, 9.3 °C).

**Table 2.** Effects of temperature oscillations on heterocyclic aromatic amine (PhIP and MeIQx; µg/kg raw sample; wet weight) levels in pork steaks and minced pork and the differences in PhIP and MeIQx levels relative to the raw pork samples. Data are means±standard deviation, where indicated (n = 120).

Oscillation amplitude (°C)	PhIP			MeIQx		
	Pork steak	Minced pork	$P_s$	Pork steak	Minced pork	$P_s$
1.2	2.11±0.46 <sup>Ea</sup>	1.15±0.12 <sup>Fb</sup>	0.045	2.66±1.34 <sup>a</sup>	0.84±0.50 <sup>Db</sup>	0.044
1.5	2.46±0.12 <sup>Eb</sup>	1.92±0.20 <sup>Eb</sup>	0.023	2.53±0.40	1.78±0.40 <sup>BC</sup>	0.152

1.6	3.81±0.32 <sup>Da</sup>	2.22±0.29 <sup>Eb</sup>	≤ 0.001	4.34±0.57 <sup>a</sup>	2.15±0.51 <sup>BAb</sup>	0.016
1.7	3.88±0.21 <sup>D</sup>	3.67±0.30 <sup>D</sup>	0.333	3.68±0.79 <sup>a</sup>	1.49±0.37 <sup>BCDb</sup>	0.004
2.7	5.16±0.20 <sup>Cb</sup>	5.62±0.38 <sup>Ca</sup>	0.038	3.13±1.09 <sup>a</sup>	1.14±0.58 <sup>CDb</sup>	0.008
4.1	5.78±0.34 <sup>C</sup>	5.35±0.52 <sup>C</sup>	0.248	3.38±0.52 <sup>a</sup>	1.74±0.72 <sup>BCDb</sup>	0.014
5.5	5.91±0.30 <sup>Ca</sup>	4.93±0.38 <sup>Cb</sup>	0.054	3.03±0.62 <sup>a</sup>	1.48±0.65 <sup>BCDb</sup>	0.018
7.8	8.28±0.76 <sup>B</sup>	5.47±0.49 <sup>C</sup>	0.015	4.86±2.13	2.85±0.73 <sup>A</sup>	0.085
9.3	10.20±0.38 <sup>Aa</sup>	7.69±0.88 <sup>Bb</sup>	0.009	3.60±1.95 <sup>a</sup>	1.41±0.76 <sup>BCDb</sup>	0.035
11.5	10.81±1.14 <sup>A</sup>	11.06±0.97 <sup>A</sup>	0.823	3.70±0.94 <sup>a</sup>	1.28±0.59 <sup>BCDb</sup>	0.004
$P_P$	≤0.001	≤0.001		0.141	0.001	
Overall matrix effect	5.84 ±2.96 <sup>a</sup>	4.91 ±2.87 <sup>b</sup>	≤0.001	3.49 ±1.25 <sup>a</sup>	1.62 ±0.75 <sup>b</sup>	≤0.001

PhIP, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine; MeIQx, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline;  $P_S$ , Statistical probability of sample matrix effect;  $P_P$ , Statistical probability of oscillation amplitude effect; Means with different superscript capital letters within columns (<sup>A-F</sup>) differ significantly ( $P \leq 0.05$ ; i.e., significance of differences between different oscillation amplitudes); Means with different superscript small letters within rows (<sup>a, b</sup>) differ significantly ( $P \leq 0.05$ ; i.e., significance of differences between pork steak and minced pork).

The data in Table 2 for the HAA levels in thermally treated pork expressed on a raw (i.e., fresh weight) basis can also be used in combination with dietary assessments to estimate the exposure to HAAs from the consumption of pork. This is because the temperature oscillations also affect the weight loss during the meat cooking, so for realistic evaluation, the data must also be defined for the raw meat. Here, the conclusions based on these data expressed as cooked weight and fresh weight are essentially the same, although the data tend to be less variable based on the fresh weights than for the thermally treated meats.

#### 4. Conclusions

The potential carcinogenicity of red and processed meat has been evaluated according to the presence of well-known/suspected carcinogenic compounds such as N-nitroso-compounds, polycyclic aromatic hydrocarbons and HAAs [3, 7]. In particular for domestic cooking of red meat, there is clearly a lack of information on the potential carcinogen compound levels and the variability of the heating conditions according to the quality of the cooking appliance used. Our findings help to address this deficiency. The present study clearly shows that talking about the recommended intake of meat in terms of these carcinogen compounds (PhIP and MeIQx) without emphasis on the preparation and appliance parameters used is essentially illusory, if not factually incorrect. Here, we have clearly proven the appropriate heating conditions and the quality of the appliance/oven used are important for producing healthier oven-roasted meats containing lower levels of the HAAs studied. Moreover, less healthy meats will result if they are prepared with a poorly regulated domestic oven.

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## Evolution of amino acids and biogenic amines in traditional dry-fermented sausage *Sjenički sudžuk* during processing

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**Abstract.** The evolution of free amino acids and biogenic amines during 23 days of drying and ripening was determined in the traditional, dry-fermented sausage, *Sjenički sudžuk*. The concentration of most amino acids increased significantly ( $P < 0.05$ ) over time, giving rise to a final concentration of total free amino acids of 600 mg/100g dry matter. Thus, the chief precursors became available for indigenous aminogenic microbiota, enabling formation and accumulation of biogenic amines. Total biogenic amines reached the level of 399 mg/kg by day 23 of processing. Putrescine and tyramine were the predominant amines. Their concentrations increased significantly ( $P < 0.05$ ) during processing, ranging from 91.9 to 212 mg/kg and from 48.5 to 147 mg/kg, respectively. A positive outcome of this study is the very low registered concentration of histamine (9.69 mg/kg), the most important amine both from the toxicological and hygienic points of view.

### 1. Introduction

*Sjenički sudžuk* is dry-fermented sausage traditionally produced in a part of the Pešter plateau, an area near the town of Sjenica, southwestern Serbia. Nowadays, this sausage is produced according to original recipes, using just basic ingredients, such as beef, salt and spices, without the use of food additives or microbial starters [1].

Throughout the fermentation and ripening of dry-fermented sausages, the proteins undergo important degradation changes, resulting in generation of numerous compounds such as polypeptides, peptides, free amino acids, aldehydes, organic acids and ammonia [2, 3, 4, 5]. Accordingly, the quantity of free amino acids, the main precursors of biogenic amines, rises during processing, which in combination with the presence of decarboxylase-positive microbiota can lead to the formation and accumulation of these compounds [3, 6, 7, 8]. Biogenic amines are organic bases that are considered negative from both the safety and hygienic points of view [8, 9, 10, 11]. Thus, it is important to monitor the accumulation of biogenic amines in fermented meat products and to try to control all the factors that can contribute to this process. Consequently, the aim of this research was to study the formation and accumulation of free amino acids and biogenic amines, during smoking, drying and ripening of traditional dry-fermented sausage *Sjenički sudžuk*.

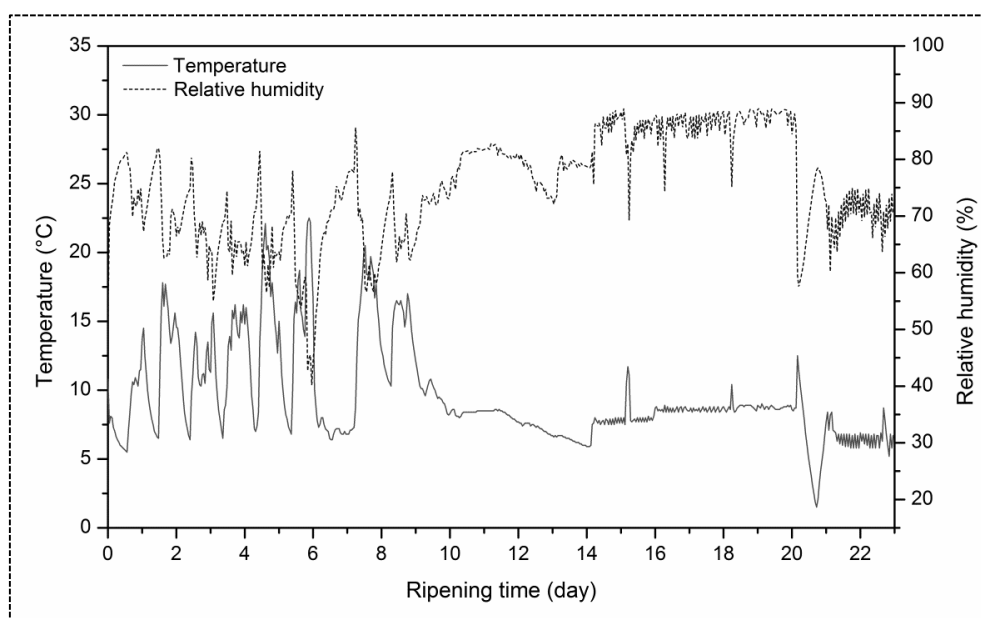


## 2. Material and methods

### 2.1. Sausage preparation and samples

*Sjenički sudžuk* were manufactured according to a traditional procedure in one small meat processing enterprise located in the town of Sjenica. Fresh boneless beef (approximately 75% lean) was salted using 35 g/kg of common salt (NaCl), and maintained at 4°C for 7 days. After this period, salted meat was ground through a 4 mm diameter mincing plate and mixed together with the other ingredients (raw garlic paste – 4 g/kg, black pepper – 3 g/kg, red sweet paprika powder – 2 g/kg), until a homogenous batter was obtained. Prepared batter was stuffed into natural casings with a diameter of approx. 40 mm and a length of approx. 50 cm. The ends of the sausages were tied off and bound together, forming a horseshoe shape. Raw sausages were entirely processed in a traditional smoking/drying room during 23 days. The environmental conditions (air temperature (°C) and relative humidity (%)) during processing are shown in Fig. 1.

For sampling, the seasoned batter prior to stuffing (0) and three randomly selected sausages were taken after 3, 7, 15 and 23 days of processing. The sausages were homogenized, vacuum packed and stored at -20°C pending analysis. Analyses for all samples were carried out in duplicate.



**Figure 1.** Environmental temperature (°C) and relative humidity (%) recorded throughout processing of *Sjenički sudžuk*

### 2.2. Determination of dry matter

Moisture content was quantified according to the ISO recommended standard [12], by heating the samples to 105°C until constant weight. Dry matter (dm) was calculated as the material remaining after removal of water.

### 2.3. Determination of free amino acids (FAA)

FAA in sausages were determined using ion exchange chromatography with utilization of Automatic Amino Acid Analyzer Biochrom 30+ (Biochrom, Cambridge, UK), according to Rabie et al. [6], with several modifications. Briefly, 20 ml of 10 % (v/v) trichloroacetic acid was added to 3 g of sample, the mixture was homogenized using a T18 Basic Ultra Turrax (IKA-Werke GmbH & Co. KG), and the extract was filtered through filter paper (FiltaTech, Fleury-les-Aubrais, France). The extracts were then centrifuged at 7000 g for 15 min using a centrifuge 5804 R (Eppendorf, Hamburg, Germany).

The supernatant was finally collected and filtered through 0.22 µm pore size PTFE filter (Plano, Texas, USA), and the filtrate obtained was transferred to an HPLC vial (Agilent Technologies, USA). FAA contents were determined by reaction with ninhydrin (Biochrom, Cambridge, UK), with photometric detection at 2 wavelengths, 570 nm and 440 nm (for proline), and expressed as mg 100 g<sup>-1</sup> of dry matter.

#### 2.4. Determination of biogenic amines (BA)

BA (tryptamine, phenylethylamine, putrescine, cadaverine, histamine, tyramine) were determined according to the procedure described by Ikonić et al. [4]. BA were determined as their dansyl derivatives, using liquid chromatography (Agilent 1200 series), equipped with a diode array detector (DAD), Chemstation Software (Agilent Technologies), a binary pump, an online vacuum degasser, an auto sampler and a thermostated column compartment, on an Agilent, Eclipse XDB-C18, 1.8 µm, 4.6 × 50 mm column. Solvent gradient was performed by varying the proportion of solvent A (acetonitrile) to solvent B (water) as follows: initial 50% B; linear gradient to 10% B in 7.6 min, 10% B to 10 min; linear gradient to 50% B in 2 min. The system was equilibrated 3 min before each analysis. Flow rate was 1.5 mL/min, column temperature was 40°C and 5 mL of sample was injected. BA contents are expressed as mg kg<sup>-1</sup> of sample.

#### 2.5. Statistical analyses

One way (ANOVA), Post-hoc (Duncan test) was performed using the software package Statistica 13.3 (TIBCO Software Inc., Palo Alto, Ca, USA). Differences were considered significant at  $P < 0.05$ .

### 3. Results and discussion

Changes in the concentration of FAA during the processing period are depicted in Table 1. The total FAA concentration in the raw sausage batter was 388 mg/100g dm. Throughout 23 days of drying and ripening, an increase in the concentration of most amino acids was registered, giving significant rise ( $P < 0.05$ ) to a final concentration of total FAA of 600 mg/100g dm. The amino acids that primarily contributed to this increase during ripening were glutamic acid, leucine and valine followed by threonine, lysine and phenylalanine. Gradual release of amino acids during ripening is characteristic in dry-fermented sausages. The registered increasing trend of FAA in *Sjenički sudžuk* is in accordance with previously reported findings by a number of authors [2, 5, 6]. On the contrary, the concentrations of serine, tryptophan and arginine decreased significantly ( $P < 0.05$ ), which could indicate more intense uptake and conversion to BA of these compounds by bacteria, than their production during ripening [2, 6].

The predominant FAA in *Sjenički sudžuk* were glutamic acid (60.8-150 mg/100g dm), alanine (69.8-81.8 mg/100g dm), serine (109-81.6 mg/100g dm) and leucine (16.8-55.1 mg/100g dm). In sum, they accounted for about 65% of the total FAA, both in the raw sausage batter and in dried sausage. These results confirm previous reports regarding the highest prevalences of glutamic acid [13] and alanine [14] in fermented sausages. Also, they differed partly from results obtained by Rabie et al. [6], who found the highest concentration of alanine, aspartic acid and glycine in beef sausage after 28 days of storage, and those reported by Domínguez et al. [5] on non-started (i.e., no starter culture) dry-fermented foal sausage, who observed leucine, cysteine and phenylalanine as the chief FAA.

Lysine, histidine, arginine, phenylalanine and tyrosine are the main precursors of dietary BA, tyramine, putrescine, cadaverine, histamine and phenylethylamine [6, 7]. Due to proteolytic changes, all of these BA, except tyrosine, significantly increased ( $P < 0.05$ ) during drying and ripening of *Sjenički sudžuk* (Table 2). FAA availability in combination with activity of decarboxylase-positive microbiota can lead to formation and accumulation of BA in fermented sausages [3, 6, 7, 8]. This fact also appears to hold true in the case of *Sjenički sudžuk*. As can be seen from Table 2, the concentration of total BA ranged from 0 to 399 mg/kg, and this was lower than the maximum threshold of 1000 mg/kg, which is considered dangerous for human health [4, 6, 9]. The relatively low level of total BA



detected during the processing period was most likely the consequence of unfavorable conditions for growth and activity of aminogenic microbiota (low temperature; Fig. 1) [3, 4].

**Table 1.** Changes in concentration (mg/100g dm) of individual and total free amino acids in *Sjenički sudžuk* during processing (mean  $\pm$  standard deviation)

Amino acid	Processing time (day)				
	0	3	7	15	23
Aspartic acid	7.27 $\pm$ 0.58 <sup>a</sup>	6.85 $\pm$ 0.31 <sup>a</sup>	9.94 $\pm$ 1.48 <sup>a</sup>	13.8 $\pm$ 2.16 <sup>b</sup>	15.0 $\pm$ 3.73 <sup>b</sup>
Threonine	7.54 $\pm$ 0.64 <sup>b</sup>	12.6 $\pm$ 3.04 <sup>b</sup>	19.2 $\pm$ 6.48 <sup>a</sup>	23.5 $\pm$ 0.75 <sup>a</sup>	25.4 $\pm$ 1.59 <sup>a</sup>
Serine	109 $\pm$ 12.8 <sup>a</sup>	104 $\pm$ 3.64 <sup>a</sup>	96.5 $\pm$ 6.48 <sup>ac</sup>	83.7 $\pm$ 1.38 <sup>b</sup>	81.6 $\pm$ 7.93 <sup>b</sup>
Glutamic acid	60.8 $\pm$ 5.03 <sup>b</sup>	86.9 $\pm$ 6.59 <sup>c</sup>	109 $\pm$ 6.35 <sup>d</sup>	141 $\pm$ 8.71 <sup>a</sup>	150 $\pm$ 8.12 <sup>a</sup>
Proline	3.92 $\pm$ 0.30 <sup>c</sup>	11.2 $\pm$ 0.43 <sup>a</sup>	11.1 $\pm$ 0.56 <sup>a</sup>	12.5 $\pm$ 1.10 <sup>b</sup>	12.4 $\pm$ 0.19 <sup>b</sup>
Glycine	11.3 $\pm$ 0.25 <sup>b</sup>	12.8 $\pm$ 1.43 <sup>b</sup>	15.9 $\pm$ 1.50 <sup>a</sup>	17.8 $\pm$ 1.41 <sup>a</sup>	18.9 $\pm$ 2.80 <sup>a</sup>
Alanine	69.8 $\pm$ 6.02 <sup>a</sup>	75.3 $\pm$ 10.3 <sup>a</sup>	79.5 $\pm$ 7.52 <sup>a</sup>	82.4 $\pm$ 9.06 <sup>a</sup>	81.8 $\pm$ 3.92 <sup>a</sup>
Cysteine	16.9 $\pm$ 0.64 <sup>a</sup>	18.4 $\pm$ 3.33 <sup>a</sup>	20.2 $\pm$ 2.42 <sup>a</sup>	21.7 $\pm$ 4.13 <sup>a</sup>	22.9 $\pm$ 4.03 <sup>a</sup>
Valine	12.7 $\pm$ 2.38 <sup>a</sup>	17.0 $\pm$ 1.40 <sup>a</sup>	25.5 $\pm$ 2.04 <sup>c</sup>	32.1 $\pm$ 2.45 <sup>b</sup>	34.7 $\pm$ 4.12 <sup>b</sup>
Methionine	9.12 $\pm$ 0.97 <sup>a</sup>	10.1 $\pm$ 1.71 <sup>a</sup>	13.4 $\pm$ 1.98 <sup>ab</sup>	15.9 $\pm$ 4.47 <sup>b</sup>	16.4 $\pm$ 1.16 <sup>b</sup>
Isoleucine	11.9 $\pm$ 0.99 <sup>a</sup>	11.5 $\pm$ 0.90 <sup>a</sup>	16.6 $\pm$ 2.90 <sup>b</sup>	20.1 $\pm$ 4.23 <sup>bc</sup>	21.3 $\pm$ 0.79 <sup>c</sup>
Leucine	16.8 $\pm$ 0.50 <sup>a</sup>	25.6 $\pm$ 5.56 <sup>a</sup>	41.4 $\pm$ 7.43 <sup>c</sup>	51.6 $\pm$ 3.66 <sup>b</sup>	55.1 $\pm$ 4.42 <sup>b</sup>
Tyrosine	1.33 $\pm$ 0.50 <sup>a</sup>	1.57 $\pm$ 0.62 <sup>a</sup>	1.73 $\pm$ 0.83 <sup>a</sup>	1.61 $\pm$ 0.56 <sup>a</sup>	1.61 $\pm$ 0.36 <sup>a</sup>
Phenylalanine	3.43 $\pm$ 0.91 <sup>a</sup>	6.52 $\pm$ 1.24 <sup>a</sup>	12.6 $\pm$ 1.79 <sup>c</sup>	16.4 $\pm$ 2.99 <sup>b</sup>	17.5 $\pm$ 2.58 <sup>b</sup>
Histidine	5.61 $\pm$ 0.30 <sup>b</sup>	6.49 $\pm$ 0.69 <sup>b</sup>	9.40 $\pm$ 0.99 <sup>a</sup>	10.8 $\pm$ 2.83 <sup>a</sup>	11.1 $\pm$ 0.94 <sup>a</sup>
Tryptophan	10.7 $\pm$ 0.52 <sup>c</sup>	8.49 $\pm$ 0.57 <sup>d</sup>	3.81 $\pm$ 0.20 <sup>c</sup>	1.92 $\pm$ 0.14 <sup>b</sup>	0.50 $\pm$ 0.19 <sup>a</sup>
Lysine	14.6 $\pm$ 1.13 <sup>a</sup>	13.1 $\pm$ 0.90 <sup>a</sup>	22.0 $\pm$ 2.92 <sup>c</sup>	28.0 $\pm$ 3.75 <sup>b</sup>	30.6 $\pm$ 2.58 <sup>b</sup>
Arginine	15.0 $\pm$ 2.85 <sup>b</sup>	15.0 $\pm$ 1.97 <sup>b</sup>	8.03 $\pm$ 0.78 <sup>c</sup>	3.07 $\pm$ 0.54 <sup>a</sup>	3.94 $\pm$ 0.42 <sup>a</sup>
Total AA	388 $\pm$ 11.2 <sup>b</sup>	443 $\pm$ 5.54 <sup>c</sup>	516 $\pm$ 40.6 <sup>d</sup>	578 $\pm$ 45.7 <sup>a</sup>	600 $\pm$ 1.16 <sup>a</sup>

<sup>a-d</sup> Means within the same row not followed by common letters differ significantly ( $P < 0.05$ )

Putrescine was the most abundant amine found in *Sjenički sudžuk*. The putrescine concentration increased significantly ( $P < 0.05$ ) during the ripening period, ranging from 91.9 to 212 mg/kg. This result is in accordance with previous reports regarding putrescine concentration in dry fermented sausages [6, 15]. Conversely, the putrescine level found in *Sjenički sudžuk* is higher than those determined in non-started sausages by Latorre-Moratalla et al. [7] and Domínguez et al. [5], and lower than levels obtained by Roseiro et al. [3] in Portuguese traditional, dry-fermented sausage. Putrescine concentration can be used as an indicator of raw material and/or manufacturing practice hygiene, since its accumulation is related to the activity of contaminant bacteria, such as *Enterobacteriaceae* [3, 8].

Tyramine was the second most common amine found in *Sjenički sudžuk* (147  $\pm$  8.30 mg/kg) after 23 days of drying and ripening, confirming previously reported findings regarding its high abundance in dry-fermented sausages. Tyramine concentration is closely related to lactic acid fermentation, due to the high potential of many lactic acid bacteria for tyrosine decarboxylation [3, 8, 11]. This amine is directly influenced by the level of tyrosine, which remained essentially constant (Table 1), indicating that on release, this amino acid was used for metabolic reactions by the sausage microbiota, and formation of tyramine resulted [6, 7].

With respect to histamine, the most important BA from the toxicological and hygienic aspects, a slight accumulation was registered after 23 days, amounting 9.69 mg/kg. Thus, the concentration of histamine in *Sjenički sudžuk* was much lower than its allowable limit in food (100 mg/kg) [5, 6, 8],

and much lower than the level reported by EFSA [16] for European fermented sausages (approx. 25 mg/kg).

Moreover, the sum of vasoactive amines (histamine, tyramine, tryptamine, phenylethylamine) did not exceed 200 mg/kg, indicating good hygienic conditions and application of good manufacturing practice during the processing of this *Sjenički sudžuk* [10].

**Table 2.** Changes in concentration (mg/kg) of individual and total biogenic amines in *Sjenički sudžuk* during processing (mean  $\pm$  standard deviation)

Biogenic amine	Processing time (day)				
	0	3	7	15	23
Tryptamine	ND	ND	ND	ND	ND
Phenylethylamine	ND <sup>a</sup>	36.0 $\pm$ 1.33 <sup>b</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>
Putrescine	ND <sup>a</sup>	ND <sup>a</sup>	91.9 $\pm$ 6.24 <sup>b</sup>	190.4 $\pm$ 0.98 <sup>c</sup>	212 $\pm$ 10.1 <sup>d</sup>
Cadaverine	ND <sup>a</sup>	ND <sup>a</sup>	20.4 $\pm$ 2.12 <sup>b</sup>	25.3 $\pm$ 0.68 <sup>c</sup>	30.8 $\pm$ 3.76 <sup>d</sup>
Histamine	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	ND <sup>a</sup>	9.69 $\pm$ 0.70 <sup>b</sup>
Tyramine	ND <sup>a</sup>	48.5 $\pm$ 2.91 <sup>b</sup>	102.8 $\pm$ 3.61 <sup>c</sup>	138 $\pm$ 3.60 <sup>d</sup>	147 $\pm$ 8.30 <sup>e</sup>
Total BA	ND <sup>a</sup>	84.4 $\pm$ 4.24 <sup>b</sup>	227 $\pm$ 12.0 <sup>c</sup>	353 $\pm$ 5.26 <sup>d</sup>	399 $\pm$ 22.9 <sup>e</sup>

<sup>a-d</sup> Means within the same row not followed by common letters differ significantly ( $P < 0.05$ )

#### 4. Conclusion

A significant increase ( $P < 0.05$ ) in the concentration of total FAA was registered during the drying and ripening period (23 days) of *Sjenički sudžuk*. The main FAA in the final product were glutamic acid (150 mg/100g dm), alanine (81.8 mg/100g dm), serine (81.6 mg/100g dm) and leucine (55.1 mg/100g dm). Along with gradual release of FAA, the formation and accumulation of BA was observed. Putrescine and tyramine were the predominant BA registered, indicating that on their release, the chief precursors, tyrosine and arginine, were used for metabolic activity by the aminogenic microbial population present. The registered histamine concentration (9.69 mg/kg) is much lower than recommended by EU regulations, which is considered a very positive finding of this research.

#### Acknowledgement

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# Challenges in analyzing polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) in food and feed in the light of the considerable tolerable weekly intake reduction proposed by EFSA in 2018

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**Abstract.** In November 2018, EFSA published a major risk assessment on the presence of dioxins and dioxin-like polychlorinated biphenyls in feed and food, proposing updated tolerable weekly intake of 2 pg/kg b.w./week, which is a 7-fold decrease over the previous value. This will inevitably result in lowering maximum levels and action levels in food and feed. This paper reflects on the possible consequences of such changes in respect to analytical capabilities in general, and the effort required to ensure suitable performance of the presently available analytical methods. Considerations related to both instrumental and sample preparation aspects are presented, taking into account specific EU legislation in the area of analytical requirements and quality control of analytical methods for dioxins and dioxin-like polychlorinated biphenyls. From the current perspective, it is obvious that any linear decrease of maximum levels and action levels is not feasible and would inevitably require some degree of mitigation of quality control requirements, at least for some food matrices that already have low maximum levels. A sustainable response to tolerable weekly intake decrease will most likely be the combination of gradual decrease of regulatory limits where achievable, inevitable technical progress in analytical instrumentation and adjustment of sample preparation techniques.

## 1. Introduction

There are several reasons for considering quantitative analysis of polychlorinated dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) in food and feed as one of the most challenging tasks in food/feed analytical chemistry. The extreme toxicity of some congeners resulting in low-ppt maximum levels (MLs), the necessity to achieve reliable measurements of levels even lower than MLs for the purposes of exposure assessments, stringent legally binding analytical and technical requirements in respect to analytical methods and the financial burden related to establishing and maintaining both instrumental and human resources involved, are just some of the hurdles inherent to PCDDF/dl-PCB analysis.

Basic principles regarding sample preparation and instrumental analysis of PCDDF/dl-PCBs in food/feed are straightforward and have not changed much in the past 30 years. Relatively large size



(e.g. 5-20 g) of raw or previously concentrated sample is extracted in organic solvents, extract is purified chromatographically using several adsorbents in manual or automated systems, and further concentrated to low volumes (e.g. 15-25  $\mu$ L) prior to injection. An array of internal standards ( $^{13}\text{C}_{12}$  substituted congeners of different chlorination levels) are added at various stages of the sample preparation process to facilitate quantification (isotope dilution method), calculation of recovery rates and estimation of extraction and cleanup efficacy. A scientifically and legally recognized confirmatory technique is capillary gas chromatography (GC) coupled to high resolution mass spectrometry (HRMS) (or tandem mass spectrometry [MS/MS] in the cases of compliance assessments as of 2014 in the EU [1]). In the case of HRMS, selectivity is achieved through GC separation of congeners and utilization of internal standards as RT identifiers, while confirmation is accomplished by high resolution ( $R \geq 10000$ ) multiple ion detection of at least two ions of isotopic cluster of the molecular ion, and verification of known isotopic ratios characteristic of chlorinated compounds. Calculated concentrations of individual congeners are corrected with respect to their toxicity by multiplication with an appropriate toxic equivalency factor (TEF [2]), and the sum of individual normalized concentrations is calculated to produce toxic equivalency (TEQ), used for determination of sample compliance.

MLs of PCDDFs and dl-PCBs expressed in ng WHO-PCDDF-TEQ/kg (ng WHO-PCDDF-PCB-TEQ/kg) are set by European legislation in food [3] and feed [4]. In 2013, action levels were introduced within the EU [5] as an early-warning tool for the purposes of preventing contamination outbreaks on a large scale, which has happened worldwide on several occasions [6]. Current MLs for PCDDF and dl-PCBs are based on tolerable weekly intake (TWI) of 14 pg TEQ/kg b.w./week, proposed by EFSA in 2001 [7]. Having in mind that existing MLs were (in absolute terms) among the lowest in the entire food/feed contaminants area, analytical methods employed for quantitative determination were developed and validated, predominantly at, and around MLs for any given matrix in most routine official or commercial laboratories analyzing samples for the purposes of official controls. Furthermore, the EU imposed stringent requirements regarding quality control of PCDDF/dl-PCB methods [1]. One of the basic requirements securing reliable quantitation at low levels is the provision that the limit of quantification (LoQ) for each congener must not be less than 1/5 of the respective ML. In the case of PCDDF-dl-PCBs analysis, LoQ is not only the method performance indicator and validation parameter: it actively contributes to the final analytical result, given the legal requirement for upper-bound approach for non-detected congeners in the expression of results [3,4]. Therefore, congener-specific LoQ determination based on legal requirements is of the utmost importance for any PCDDF/dl-PCB analytical method used for the purposes of official analysis. Its overestimation can lead to unrealistically high TEQ values and false non-compliances, while its underestimation can (in borderline cases) lead to the issuing of a false compliant result. However, the upper-bound approach is basically an additional consumer protection measure from the toxicological perspective, given the high toxicity of PCDDF/dl-PCBs. The whole system established in this way proved to be sustainable, and an increasing number of official laboratories took part in dioxin analysis, successfully employing analytical methods and demonstrating competence by participation in numerous proficiency tests.

However, in 2018, EFSA published its “first comprehensive risk assessment of dioxins and dioxin-like PCBs in food and feed, reducing the tolerable weekly intakes seven-fold (2 pg TEQ/kg b.w./week), based on new data and methods and indicating a health concern due to exceedance of the new TWI across the EU population” [8]. This will inevitably lead to lowering of MLs and action levels to a degree yet to be announced, consequently having a significant impact on current performances of analytical methods.

The aim of this paper is to reflect on the consequences of EFSA’s recommendations on the PCDDF/dl-PCB analytical methodology, from the instrumental, sample preparation and systemic aspects, and to propose possible strategies for arriving at a solution for the challenging task put before the dioxin analytical community and regulatory bodies Europe-wide.

It is the opinion of the authors that there are three possible areas where improvements could lead to lowering of MLs: 1. Improvements in current analytical methods with respect to sample preparation; 2. Future improvements in instrumentation design and capabilities, and; 3. Systematic measures e.g. participation in proficiency tests designed to target PCDDF/dl-PCB concentrations lower than current MLs, and joint efforts of official laboratories coordinated by the European Reference Laboratory (EURL) in further method refinements.

## **2. Improvements in sample preparation methods**

The introduction section of this paper outlines the sample preparation steps in PCDDF/dl-PCB analysis. The most obvious way to lower the limit of detection (LoD) or LoQ of any method is to increase sample size and/or the degree of concentration of the final extract. The latter is not easy to accomplish, since final extract volumes are already very low (15-25  $\mu\text{L}$ ) and further reduction will seriously impair manipulation of the extract, thus leading to considerable errors. Although extensive quality control is incorporated in the methods (isotope dilution quantification, series of standards for extraction efficiency calculation and calculation of recovery rates) to provide a high degree of robustness, final volume misinterpretation due to technical difficulties in manipulation of the extract (e.g. precise evaporation, rinsing the vessel walls) will inevitably lead to significant analyte losses and, consequently, the inability to meet prescribed recovery rates.

The situation in the case of sample quantity increase is theoretically more promising. It would be the first and obvious choice of any analyst when there is a demand for higher sensitivity. However, there are several limitations in this approach.

Current methods designed for modified PCDDF/dl-PCBs analysis in food/feed already take into account the relatively high sample weights, and the equipment used in laboratories for extraction and cleanup steps is adequately sized for that purpose. In order to meet several-fold decrease in MLs, the increase in sample weight would be considerable, which would lead to replacement of extraction/cleanup systems with physically larger equipment, increase in consumption of solvents and adsorbents and higher costs of analysis. In the cases of automated sample preparation systems, producers of such equipment would have to develop different sizes or types of adsorbents capable of dealing with increased sample loads. All this would undoubtedly pose significant financial burden.

A better strategy would be to test, in a systematic and organized manner, the existing sample preparation capacities for accepting increased sample loads, especially manual glass chromatographic equipment (increased adsorbents quantity, alterations in elution, solvent selection etc.). If proved feasible, some degree of sensitivity increase would certainly be achieved, although not in the order of magnitude required.

On the other hand, increased sample size without adequately increased cleanup efficiency would introduce more interference in the extraction and could result in a reverse effect, raising the PCDDF/dl-PCBs levels in blanks and, consequently, increasing LoQ values. According to internationally recognized analytical methods [9], the actual limitations in dioxin analysis are related to the (in)ability to produce sufficiently low contaminated pseudo-blanks, rather than any sensitivity deficiency of HRMS. Instrumental LODs of modern sector instruments are in the concentration ranges of low  $\text{pg/L}$ .

One more approach is perhaps worth considering, regarding existing method performance. PCDDF/dl-PCBs analysis is, as already mentioned, heavily regulated in many respects. The legal requirement for the LoQ to be 1/5 (or less) of the respective ML is one of the key guidelines during method evaluation and validation. In some instances (i.e. for some matrices for which higher MLs have been set), due to inertia, laboratories might have only verified this requirement rather than tested it, leaving reported LoQ at or around the required limit. This would mean actual LoQs are realistically lower than are reported by those laboratories. If this is proved correct, revalidation of existing methods would yield some increase in method performances, although it would be unrealistic to expect significant lowering of the existing LoQs. Reasons for this are twofold: firstly, this “underestimation” of the method performance cannot be large, and hence, the contribution of re-evaluation would not be

significant. Secondly, there are already matrices (e.g. infant formulas) where MLs are extremely low in the current legislation, and where it is evident that the “1/5 of ML stipulation” cannot be met, even today [10].

### 3. Improvements in technical aspects of instrumentation

This is by far the best and obvious approach when there is a demand for increase in analytical capabilities. However, this process cannot be governed administratively, while the results of such changes cannot be held to any predicted or imposed timeline. Mass spectrometry has rapidly expanded, becoming an analytical technique of choice in the past decade, especially in the area of official analysis of food/feed. High resolution instruments having other than sector type analyzers, are already on the global market at competitive prices. Although they currently lack the sensitivity needed for PCDDF/dl-PCBs detection, it stands to reason that in the future they might compete with, or even replace the “gold standard” in dioxin analysis. Magnetic sector instrumentation is currently the dominant technique, but nevertheless, it suffers from several deficiencies in the practical, rather than the analytical domain. The price of these sector instruments is still among the highest in the field of routine mass spectrometry. Furthermore, their size, limited number of manufacturers, limited service support (compared to e.g. ubiquitous quadrupole instruments), steeper learning curve for the analyst and more complex/demanding skills needed to operate and maintain such instruments, are still impeding factors especially in high-throughput, routine, official or commercial laboratories.

Together with approval by the European Commission in 2014 of MS/MS instruments for official controls at the levels (MLs) of interest, it seemed that magnetic sector instrumentation, as providing the technique of choice for PCDDF/dl-PCBs analysis, would become a somewhat smaller niche within the reference laboratories and research centers. The language used in current Commission Regulation (EU) 2017/644 and its predecessor (589/2014) is cautious and balanced, limiting the use of MS/MS to compliance assessment only, while leaving exposure assessment (background contamination) to HRMS exclusively. Having in mind the magnitude of the TWI decrease proposed by EFSA in 2018, and the almost certain significant lowering of the MLs by the European Commission, the analytical community might be faced with the situation in which today’s background levels would be close to or even surpass tomorrow’s regulatory levels (one should not forget that action levels are regulatory as well). Other researchers [11] involved in exposure assessments in humans have demonstrated that new sector instruments are technically capable of reaching as low as atogram levels on columns, for analysis of human plasma and milk samples. However, this level is not achieved without difficulties and with questionable reproducibility, even in a laboratory far beyond any routine food/feed control facility.

Regulatory analytics is the field where predetermined stipulations have to be met, and it is obvious that a considerable amount of time and effort will be required in order to achieve several-fold lower levels on a routine basis. On the other hand, in the case of MS/MS analyzers, the question of the competency of current instruments with respect to the required sensitivity for enforcement purposes has to be raised. That question is not addressed solely to the analytical community; it is a legal question *par excellence*. What would be expected from the analytical community is to demonstrate (or not) the ability of such instruments to comply with the new requirements, without any mitigation in regulatory requirements. The competent authority i.e. the European Commission, would have to respond if the outcome of the testing proves to be non-satisfactory.

Undoubtedly, technical progress will, at some point, deem MS/MS instrumentation fit for use in PCDDF/dl-PCBs analysis at the new levels, as was the case with current requirements.

Another consideration is worth mentioning: for obvious reasons, the European Commission could not endorse or impose any particular producer and/or model of instruments. That is not an issue in the sector, since any commercial manufacturer offers a singular model, which is by default capable of reaching the required MLs (in general, magnetic sector instruments are traditionally used for regulatory purposes only in limited areas of analysis of halogenated persistent organic pollutants (POPs), sport doping control and certain petrochemical applications). However, in the much broader

realm of MS/MS, a considerably wider gamut of instruments are available in any manufacturer's portfolio, and they are not all capable of performing PCDDF/dl-PCB analysis. In the light of potential considerable ML reductions, an even lower number of such instruments will qualify for this task. Naturally, manufacturers themselves advertise their products for certain applications. However, the plethora of available models might cause a degree of confusion or even misuse. Since having the regulators endorse the equipment is not an option, recommendations by the scientific community or reference laboratories would be advisable.

#### **4. Systematic measures within the framework of the EU Reference laboratory**

Probably the most important set of measures to be taken before the considerable decrease in MLs of PCDDF/dl-PCBs is brought into force is to demonstrate and ensure the feasibility of such actions in the long term. The role of the EURL for Halogenated POPs would certainly be crucial in that domain. A network of National Reference and official laboratories within the EU has been established, and coordination between laboratories by the EURL in identifying and conducting the necessary steps in adjusting to the much lower MLs is of great importance.

EURL is also the organizer of the proficiency testing schemes that provide the most accurate insight into individual laboratory competency. However, due to the nature of PCDDF/dl-PCBs analysis, i.e. very low MLs, most of the proficiency testing schemes are conducted with samples contaminated at or around the current MLs. Therefore, it would be necessary to re-assess the ability of laboratories to perform at lower levels through several cycles of proficiency testing where samples are contaminated at lower levels, in accordance with the TWI published by EFSA.

Another role of the EURL in this matter would be in the coordination of efforts to modify or adjust analytical methods in order to lower existing LoQs and to govern the testing of such modifications throughout the network. Having in mind that current MLs also vary considerably between matrices, it would be safe to assume that some "high" MLs (e.g. fish oils) could be reduced with no additional effort, since the existing lower-end working range would allow it without modifications of the actual method or re-validation. However, many of the matrices would have to be thoroughly revisited, which would require a considerable amount of time and funds. Hence, it will be essential that those efforts are efficiently coordinated by the EURL.

#### **5. Conclusion**

It is evident that the review on risk to human and animal health from dioxins and dioxin-like PCBs in feed and food, published by EFSA in November 2018, will result in considerable decrease of current MLs for these contaminants. This poses a challenge for the analytical community in conforming to the new, stricter requirements without sacrificing any of the criteria and requirements for quality control of the analytical methods. This adjustment process will not be rapid, and cannot be implemented without considerable obstacles. For that reason, it is important the adjustment is conducted systematically and supervised by the EURL at all stages. Some of the current MLs can be lowered immediately, but others will require time, scientific and technical effort and some funds to be accomplished. Having in mind that stringent analytical and regulatory requirements in dioxin analysis are the cornerstones of reliable analytical results at ultra-trace (low ppt) levels, a sustainable response to TWI decreases would most likely be the combination of gradual decrease of regulatory limits where achievable, inevitable technical progress in analytical instrumentation and adjustment of sample preparation techniques, all under the supervision and coordination of the EURL.

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## Zinc migration into liver pâté and pâté with ham packaged in black colored polypropylene containers

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**Abstract.** The European food contact legislation requires verification of compliance for migration of substances from plastics food contact materials with both overall and specific migration limits. To do so in this study, migration tests were carried out using food simulant and food under well specified time and temperature conditions. A total of 28 samples of liver pâté or pâté with ham packaged in black polypropylene containers were examined for the presence of Zn according to Serbian regulation after 14 months' storage from their production dates, and data were compared to results obtained by testing empty, unused containers according to EU legislation. Elevated Zn concentrations of 161.27-327.54 mg/kg (above the maximum prescribed limit of 100 mg/kg) were registered in pâté from zones in contact with the polypropylene packaging, while pâté in zones not in contact with packaging had lower Zn levels (9.98-15.08 mg/kg).

### 1. Introduction

Migration is a global term to describe a net mass transfer of chemical substances from a packaging material into the food [1]. Migration of substances from food contact materials to food must not occur in amounts that endanger human health. Migration tests can be divided into two distinct phases. The first is the migration exposure itself, i.e. the contact of the plastic material to the food simulant. The second is the quantification of the migrants by chemical analysis for specific migration and by gravimetric analysis for overall migration [2].

In many cases, migration is governed by a mass transfer process called diffusion that can be described by Fick's law:

$$\frac{\partial c}{\partial t} = D_p \frac{\partial^2 c}{\partial x^2}$$

where C is the concentration of migrant in the food contact material or article (P) at Time t and at distance x from the origin of the x-axis, and D<sub>p</sub> is the diffusion coefficient in the food contact material or article. In practice, a monolayer, homogenous, plastic food contact material or article (P) can be regarded as a film or sheet of finite and constant thickness (D<sub>p</sub>) being in contact with food of finite volume (VF) and contact area (A). It is assumed that at the time of bringing the migrant into contact



with food ( $t = 0$ ), the migrant is distributed homogeneously in the plastic [3]. Therefore, the key parameter necessary for migration modeling is the diffusion coefficient of the migrant in the plastic. One problem is that for some metals, knowledge on actual release of metal ions is limited, and hence, no inorganic compounds, metals, metal oxides etc. are eligible to have their migration into the packaged food product modeled. [4] To aid industry and national food authorities, the Council of Europe has suggested specific release limits (SRLs), and for Zn, this is = 5mg/kg [5]. Zn is a nutrient that plays many vital roles in mammal bodies [6], but that in higher concentrations can be toxic. The main role of food simulants is that they can be used for estimating migration/release, but food simulants will not always provide correct estimation of the release of metal ions from a food contact material to food.

The main goal of this case study (Zn migration into meat pâté products) was to demonstrate in practice the above-mentioned claim that the results of the previous testing of the packaging should be taken with some reservation if the intention is to keep the product for a prolonged time.

## 2. Materials and methods

### 2.1. Samples

Empty, unused polystyrene packaging containers, 90 ml (Figure 1) were filled to the top with and exposed to olive oil for 10 days at 60°C. The analysis of metals was performed according to SS EN ISO 17294-1,2 (modified) and EPA method 200.8 (modified) using ICP-SFMS technique. These data were supplied by a laboratory based in Sweden.



**Figure 1.** Examined container for pâté made from cast black colored polypropylene



**Figure 2.** Discoloration of liver pâté surface that had been in contact with the polypropylene container

A total of 28 samples of liver pâté or pâté with ham were examined for the presence of Zn (Table 2) at 14 months after their production dates and storage at up to 25°C, when visible discoloration of the liver pâté surfaces was visible *via* experimental, periodic visual controls. For analysis, samples of the two different pâtés were taken in an identical manner from two different zones (inner layer of pâté not in contact with the packaging and pâté layer with visible discoloration in contact with the packaging container wall). Pâté samples were homogenized and then prepared for instrumental analysis.

The Zn content of these test portions of the two kinds of pâté were obtained in the manner described above for the packaging. Briefly, test portions were mineralized by adding 5 mL of 65% HNO<sub>3</sub> and 1.5 mL of 30% H<sub>2</sub>O<sub>2</sub> (Merck, Darmstadt, Germany). Microwave assisted digestion was performed in START D (Milestone, Italy). The following temperature program was used (default food program): 5 min – ramp up from room temperature to 180 °C; 10 min hold at 180 °C; 15 min cooling. After cooling, digested samples were quantitatively transferred into 100 mL polypropylene volumetric flasks and diluted to volume with ultrapure water. The analysis was performed by inductively-coupled plasma mass spectrometry (ICP-MS) using the instrument iCap Q (Thermo Scientific, Bremen,

Germany), equipped with a collision cell and operating in kinetic energy discrimination (KED) mode. The isotope measured was zinc ( $^{66}\text{Zn}$ ). Torch position, ion optics, and detector settings were adjusted daily using a tuning solution (Thermo Scientific Tune B), in order to optimize measurements and to minimize possible interferences. For the quantitative analysis of the samples, a five-point calibration curve (including zero) was constructed in the concentration range of 0.1-2.0 mg/L for  $^{66}\text{Zn}$ . An additional line of the peristaltic pump was used for online introduction of a multi-element internal standard ( $^{45}\text{Sc}$  – 10 ng/mL;  $^{71}\text{Ga}$  – 2 ng/mL). Concentrations of measured isotope were corrected for response factors of both higher and lower mass internal standards using the interpolation method. The quality of the analytical process with respect to the accuracy and precision was assessed by analysis of the standard reference material SRM 1577c (NIST, Gaithersburg, MD, USA). Reference material was prepared in a random manner during microwave digestion of each sample batch and run at the beginning, in the middle and at the end of each sample list. This method is accredited by Accreditation Body of Serbia (ATS) according to ISO/IEC 17025:2006, Accreditation number: ATS 01-049.

## 2.2. Statistics

Statistical analysis was performed by the MINITAB software package, version 16.0. Concentrations were expressed as mean values, standard deviations, median and range of minimum to maximum.

Before choosing the appropriate statistical analysis, four different individual distribution identification methods were conducted as the first step to identify the native distribution. Probability plots were used for visualization of graphical goodness of fit test. The one way ANOVA and *post hoc* Tukey's honestly significant difference test (Tukey's HSD) were used to test the significance of differences between means for the groups of samples analyzed. The differences were considered statistically significant when the p-value was less than 0.05. Interval plots were used to illustrate both a measure of central tendency and variability of the data.

## 3. Results and discussion

The contents of specific metals that migrated into the simulant, olive oil, from the new, previously unused polypropylene containers in which product can be packaged are presented in Table 1. Initially, there were no significant concentrations of the measured metals registered in the simulant used (olive oil).

**Table 1.** Heavy metal concentrations in olive oil simulant filled in previously unused polypropylene containers and stored for 10 days at 60°C

Simulant	Metal content (mg/kg simulant)				
	Zn	Cd	Cr	Hg	Pb
Olive oil	<0.4	<0.002	<0.04	<0.004	<0.008

Zinc concentrations (mg/kg) measured in different zones of canned liver pâté or pâté with ham (packaged in black polypropylene containers, hot sealed with lids made from aluminum foil, and originally top covered with [apparent] polypropylene black lids, net weight 90g) are shown in Table 2.

**Table 2.** Zinc concentrations in different zones of two pâté products packaged in PP containers

	Declared expiry date (months)	Duration of storage after production (months)	N	Zn* (mg/kg) Mean	Standard deviation	Minimum Zn level (mg/kg)	Maximum Zn level (mg/kg)	Median Zn level (mg/kg)	Range of Zn levels (mg/kg)
Liver pâté mass (inner layer)	36	14	8	16.2 <sup>B**</sup>	6.4	9.9	28.7	13.9	18.7
Ham pate mass (inner layer)	36	14	6	29.5 <sup>C**</sup>	3.5	23.7	34.6	29.7	10.9
Liver pâté mass in contact with (container wall)	36	14	8	201.6 <sup>A**</sup>	53.6	161.3	327.5	185.3	166.3
Ham pâté mass in contact with (container wall)	36	14	6	55.2 <sup>C**</sup>	8.6	41.6	70.4	54.4	28.9

<sup>n</sup> number of examined samples; \* maximum permissible concentration of Zn in the content is 100 mg/kg [7] < \*\* means that do not share a letter are significantly different (p<0.05)

Visual observation of the content was performed after removing the sealed aluminum lid from the top of the containers and vertical deliberation of the pâté onto a white plate. Pâté with ham, with production dates close to those of liver pâté and packaged in the same polypropylene containers, had no visible discoloration, but this was not the case for liver pâté. Inspection of liver pâté revealed spotted greyish-black discolorations in the form of a continual stripe, approximately 5-6 mm wide, on surfaces that were in contact with the polypropylene packaging material, immediately below the internal rim of the container. Some liver pâté samples also showed visible discolorations on the surface that had been in contact with the internal refracted bottom rim. Zn concentrations higher than maximum prescribed value (100 mg/kg as defined by Serbian regulation) were measured in liver pâté portions obtained from the zones of visible discoloration (Table 2).

The examined liver pâté samples had significantly higher (p<0.05) Zn concentrations (range from 161.27 to 327.54 mg/kg) in the product mass taken from contact zones with visible discoloration, than in the product mass taken from zones of product without discoloration (9.98-15.08 mg/kg). Pâté with ham had significantly (p<0.05) lower Zn levels both in contact (41.6-70.4 mg/kg) and in non-contact zones (23.7-34.6 mg/kg) than did liver pâté. There were no statistically significant differences (p>0.05) of measured Zn concentrations in pâté with ham derived from contact and non-contact zones.

Taking into account the identical expression of visible discoloration (geometric locus) and the much higher Zn concentrations (10 to 20 times) in contact zones than in non-contact zones, in the case of liver pâté, it can be strongly concluded that migration of Zn occurred from the curved container's wall material into the product during the 14 months' storage period. Such migration was also measured, but was not visibly detectable in pâté with ham, in which Zn concentrations were lower (maximum measured Zn concentration in the contact zone was 70.4 mg/kg) (Table 2). Lower Zn concentrations in product mass taken from contact zones could be explained due to the different formulation of this product in comparison to liver pâté [8]. Pâté with ham could contain a higher content of animal protein, and the stability of such a meat emulsion is better.

The very nature of the reaction is based on the fact that, for example, zinc stearate is an extremely polar molecule, which, when embedded in otherwise nonpolar polypropylene, leads to electrical incompatibility between the additive and the plastic material, in this case, polypropylene [9]. This assertion, first of all, takes into account that during the production of polypropylene containers, zinc-containing compounds (e.g. zinc stearate or zinc oxide) are used as additives, which have multiple roles in the process of making the packaging, such as: facilitating the removal of molds, lubrication, regulation of acidity of molten polymeric mass, improvement of pigment dispersion and dyeing, and effect on polymer rheology [10]. This Zn added to the packaging material can specifically migrate to the inner surface of the plastic material, which is, in turn, in contact with the food product.

The multiple-fold increased Zn content in the zone of product discoloration could also be associated with container geometry-related variables (thicknesses, contact area topography, volumes) as well as time and temperature of product storage (discoloration was visible where product had contact with packaging curvature) [11]. Numerous factors influence the speed of this reaction, especially pH and the nature of the food. If we suppose the pâté mass is highly polar in nature (since it contains a degree of fat), the conditions for the migration of polypropylene additives are created.

It should also be borne in mind that the pâté itself contains vegetable oil, added to improve pâté lubricity, and so the food itself becomes an exceptionally good migration medium (for example, olive oil serves as a migration simulator according to European Directive 85/572/EEC, so-called simulant D) for components used in plastic container production [12]. It is recognized that the use of metallic stearates is associated with problems with the discoloration of foods in contact with polymeric material [13]. The chemical examination of empty, unused containers (Figure 1) did not determine any malfunction of the finished product according to prescribed food simulants, accredited methods of packaging material testing or legal MRL limits (Table 1).

A general conclusion from this study is that results from migration/release testing with food should prevail over results from tests with approved simulants. Therefore, if analyses are available with the food itself and also in simulants, the results from analysis with food will be considered as documentation for packaging material compliance. Consequently, it was deemed necessary to establish guidelines to regulate these situations. The guidelines take into account the fact that some inorganic compounds, metals, metal oxides, metal salts, etc., are not eligible for modeling [14].

Results from this case study also indicate the elevated Zn concentrations in liver pâté might endanger human health if the total content of the metal in food exceeds the health-based guideline values or brings about an unacceptable change in the composition of the food or a deterioration of its organoleptic characteristics.

As a final recommendation, it is possible to review the defined shelf life and storage conditions taking into account the nature of packaging and the specific product formulation.

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# Perspectives in meat processing

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**Abstract.** Meat technology includes all steps from animal handling and slaughtering to production of different meat products. During animal handling, special attention is paid to animal welfare, not only to protect animals from suffering but also because of animal welfare's importance for meat quality. The oldest processing methods (chilling, freezing, salting, smoking and drying) are being readjusted with respect to equipment and consumer's health issues. Special attention is given to preservation of meat's nutritive value (milder heat treatment) and health promoting properties of the products (functional foods). Novel methods (irradiation, high pressure, pulsed electric field, pulsed light and cold plasma) struggle with some issues such as acceptance by consumers, expensive equipment and effects on food's sensory properties. Along with novel products, demand for traditional meat products is still increasing which requires the uniqueness and quality of these products to be preserved, along with increased production capacity.

## 1. Introduction

Meat and meat products are a very important source of nutrients in human diets. The whole process of obtaining meat reaches back to the farms and includes animal handling, transport to the slaughterhouse and slaughtering process, followed by preservation techniques aiming to extend the shelf life of fresh meat or to convert it into meat products [1]. From the oldest techniques to preserve meat such as salting, smoking and drying, the development of the meat industry led to new techniques that include contemporary equipment and machines on the one hand, and chemicals and non-meat ingredients on the other hand [2]. Along with preservation techniques, the concept of animal welfare has also flourished, not only to protect animals from suffering but also because of animal welfare's influence on meat quality [3]. The awareness of consumers about the influence of food on their health has led to the rise of the concept of functional food, which also inspired research in functional meat product development [4] as well as in preserving the nutritive value of products [5]. Apart from conventional meat processing techniques, there are novel approaches, including high pressure or irradiation, that have their advantages and disadvantages [6,7]. However, traditional meat product manufacture still retains its significance and certainly commands attention thanks to increasing demand for these products by consumers [8]. The aim of this paper is to discuss contemporary meat processing concepts and approaches and to review their current status and future perspectives.

## 2. Animal welfare and meat quality

Although *meat processing* is the "further processing of meat" [9], it would be negligent not to mention the quality issues of meat as a raw material. In the last two decades, awareness of the influence of





stress in animals intended for slaughter on meat quality has increased. The first meeting of the *Ad hoc* Group on the Humane Slaughter of Animals within the OIE (World Organisation for Animal Health) was held in 2003, which further resulted in defining guidelines for the slaughter of animals for human consumption [10]. This provoked further research into stressors and their indicators. Already during transport to the slaughterhouse, animals are exposed to stress [3], which continues in the lairage and during stunning [11]. Lairage duration and animal density influences meat quality, showing a positive correlation with the occurrence of PSE (pale soft exudative) and DFD (dark firm dry) meat [12]. The breeding system influences the occurrence of pathological lesions in slaughtered pigs [13]. Biochemical stress indicators such as lactate and acute phase proteins also show positive correlations both with stress and meat quality [14]. Improper animal stunning procedures point to the need for continuous staff training [12]. Additionally, slaughter of stressed animals leads not only to meat quality defects but also could lead to endogenous microbial contamination, endangering the safety of meat and, consequently, of meat products [15].

### 3. Conventional meat processing methods

#### 3.1. Chilling and freezing

From meat chilling in natural caves followed by building cellars cooled by natural ice [16], technological development led to mechanical refrigeration and the emphasis on processes currently used, such as quick chilling and super fast chilling by cold air, as well as spray and immerse chilling by cold water [1,16,17]. A new approach, which is a variation of super fast chilling, is *stepwise chilling*. This process includes three steps: fast chilling, tempering and fast chilling again. This approach leads to 5% energy cost savings compared to super fast chilling, but it also results in meat tenderisation because of meat enzyme activation during the tempering phase [2]. A future perspective could be so-called *pad chilling*, based on the direct contact of the animal carcass on a cold surface, whereby the temperature is lowered through the conduction process and not evaporation like in conventional chilling. This process is 30% faster, chill loss is very low (0.1-0.2%), and the energy cost saving is about 50% compared to conventional chilling methods [2]. As a newer method that is between chilling and freezing, *superchilling* involves initial freezing of the surface, then meat is exposed to equalization of ice crystals at only 1-2°C below the freezing temperature. The shelf life of such meat could be up to four times longer than that of chilled meat. Furthermore, because there is no need for any thawing process, this method reduces costs in labour, energy and weight loss, as well as resulting in meat with better sensory characteristics than frozen meat has after thawing. However, this method requires more precise temperature control and monitoring systems because of the narrow range between superchilling and chilling temperatures [18]. Freezing of meat expanded at the end of 19<sup>th</sup> century, as a method that provided long shelf life of meat and long distance transport from one part of the world to another. Although meat storage below -18°C stops all microbial growth and cellular metabolism, lipid oxidation remains the main problem during storage. Adequate packaging films could help in lowering the oxidation processes and the incidence of freezer burns on meat surfaces [1,16,17]. A relatively newer method, *cryogenic freezing* is faster than air freezing and requires only tanks or sprays for cryogen application. However, it is suitable only for smaller and packed meat pieces, and the cost of the cryogenic liquid makes this method relatively limited in commercial use [18].

#### 3.2. Salting and Curing

Salting was recognised very early in human civilisation as a method of prolonging meat's shelf life without chilling [16]. Curing also arose long ago in history, when ancient civilisations incorporated nitrates in meat via contaminated salt, but it took a long time until the actual chemical processes in cured meat were completely understood [17,19]. At present, nitrite plays a very important role in the meat industry, in the first place because of its strong antimicrobial activity, especially against *Clostridium botulinum*, as well as it being a colouring agent. Recent studies, however, proved the carcinogenic potential of N-nitrosamines that can be formed in cured meat products, which provoked a series of investigation in order to find nitrite substitutes. Promising results were obtained with

essential oils, polyphenols, lactic acid bacteria and acid whey, etc. [4], but these single ingredient solutions could provide only one of the main functions of nitrite in meat products. As it is also known that endogenous production of nitric compounds in the human body occurs physiologically from other sources of nitrate and nitrosamines not from meat products, the exclusion of the use of nitrites is not yet in sight [19]. Additionally, although some traditional meat products are produced without the use of curing salts, many of these products contain nitrate and nitrite residues which are indirectly incorporated in the products via spices [20,21]. Such products are often labelled as “uncured” or “without preservatives” which could be misleading for consumers in terms of nitrite presence in the product [19] and in terms of product labelling [22]. As for the use of NaCl, some meat products are a significant source of high sodium intake in the human diet, which is recognized as cardiovascular disease promoting factor. Partial replacement of NaCl with K, Ca and Mg salts (chloride, lactate etc.) could reduce the sodium content in meat products without adverse effects on products’ properties or safety [4,23].

### 3.3. Smoking

Smoking of foods, including meat, originated thousands of years ago and is a preservation method based on chemical compounds from smoke that influence the sensory properties of meat products and have antimicrobial and antioxidative roles on the meat surface [17,24]. Recently, emphasis has been given to the investigation of harmful smoke compounds such as polycyclic aromatic hydrocarbons (PAHs), because of their carcinogenic potential. There are several approaches in order to produce smoked meat products with less or with no PAHs, which include measures to obtain smoke with less PAHs by the controlled process of wood pyrolysis, smoke purification, or even to reduce the content of smoke in meat products by means of starter cultures or some spices [4]. The other approach to obtain smoked meat products free of PAHs is to use smoke flavourings. As the toxic compounds are not soluble in the water phase of the liquid smoke, the compounds are easily removed, producing smoke flavourings with no detectable amounts of PAHs [24].

### 3.4. Drying

Along with salting and smoking, drying is one of the oldest meat processing methods. Until the industrial revolution, meat was dried under the influence of natural climate conditions, but during the last century, the use of climate chambers became irreplaceable in the meat industry. However, as climate chambers are significant energy consumers, this fact provoked investigations on shortening the drying process or introducing new air drying systems [25]. Some approaches to shorten the drying process are microwave vacuum drying, which is a process combining microwave heating and vacuum drying [26], and ultrasonic vacuum drying [27]. Drying in a vacuum chamber is more than three times faster than drying under usual conditions, and the meat’s sensory properties are not affected, while microbiological stability is achieved more rapidly [28]. As for air drying systems, a heat pump dryer is more efficient than hot air drying systems. Among heat pump drying systems, the best appear to be CO<sub>2</sub>-heat pumps, followed by glycol heat pumps, while ammonia heat pumps were less effective [25].

### 3.5. Heat treatment

Although early humans used fire to prepare meat for consumption, the commercial thermal processing of meat started at the beginning of 19<sup>th</sup> century with Nicolas Appert and design of the first canned products [16]. Heat treatment provides safety, shelf life, sensory properties and better digestibility of meat, and, depending on the chosen temperature intensity, pasteurization, cooking or sterilization can result [5]. Hermetically sealed cans are a good environment for growth of anaerobic bacteria, with special attention paid to toxigenic *Clostridium botulinum*. Therefore, process control of canning gained its importance, which resulted in development of  $F_0$ -values. These precisely measure whether the canning process ensures meat safety with respect to *C. botulinum* ( $F_0 \geq 3$ ) [15,16,17]. Sterilized cans are usually overheated in practice ( $F_0 > 8$ ) in order to ensure product safety, but such processing significantly affects the nutritive value of the canned products. Such unnecessarily strong heat treatment could be optimized by  $F_0$ -value and  $C_0$ -value (cooking value) determinations, in order to provide safety and preserve the nutritive value as much as possible [5]. Furthermore, it is possible to

produce *shelf stable meat products* by mild heat treatment and which can be stored at room temperature, by means of the hurdle technology concept based on the simultaneous action of several antimicrobial parameters (pH, water activity, redox potential,  $F_0$ -value, temperature). This approach led to the development of the so-called *refrigerated processed foods of extended durability*, where the accent is on mild heat treatment and prolonged shelf life [15].

### 3.6 Packaging

The main purpose of packaging was firstly just to mechanically protect products from contamination, over-drying, oxidation, weight and nutrient losses, adsorbing strange odours from the environment etc., simply by overwrapping with packaging material. With the development of polymer materials with low gas permeability and the technology to remove air from the packaging, vacuum packaging emerged, which additionally provides reduced oxidative changes and limited growth of aerobic bacteria. This led further to the development of modified atmosphere packaging, which provides antimicrobial effects due to the high  $\text{CO}_2$  concentration in the packs. Another novel method is active packaging, where active components are purposefully incorporated into the packaging material. There are several variations of active packaging: *antimicrobial packaging* which releases antimicrobial substances during storage (silver ions, bacteriocins, essential oils, nisin etc.), *antioxidative packaging* containing oxygen scavengers, and finally, *intelligent packaging* which contains sensors and indicators for detection of gas, temperature abuse, package integrity etc., and which utilise colour changes to inform the consumer about the state of the products [18,29]. As active packaging is mostly based on nanotechnology, there are still issues concerning the lack of knowledge about the impact of nanomaterials on human health and their occurrence and destiny in the environment, which should be further studied [30].

## 4. Novel meat processing methods

### 4.1. Irradiation

Food irradiation was developed during the second half of the 20<sup>th</sup> century [31] and involves exposure of food to ionizing irradiation (Gamma rays, Electron beams) in order to promote safety and prolong the food's shelf life, but also refers to product quality examination using computer tomography (CT) based on X rays. Gamma rays are obtained from radioactive isotopes  $\text{Co}^{60}$  or  $\text{Cs}^{137}$  with relatively short half lives (5.27 and 30.19 years respectively), with electron beams produced in an electron accelerator and X-rays produced by slamming electrons into metal (tantalum or platinum). Irradiation dose refers to the amount of absorbed energy in Greys (Gy) [31,32]. Irradiation is a highly effective, cold, penetrative and relatively easy to control process, which does not affect the sensory or nutritive properties of food if properly used. One problem can be lipid oxidation in high fat foods, but simultaneous use of antioxidants can prevent this [31]. Although food irradiation has been approved since 1989 by the USDA and FDA, nowadays, about 30 % of countries worldwide have adopted it as a commercial method of food preservation, led by the USA, China, The Netherlands, Belgium, Brazil, Thailand and Australia. The types of foods irradiated are mostly spices, crops, vegetables, fruits etc. and to a smaller extent, meat, mostly ground beef, poultry, fish and seafood. The main obstacle for wider use is acceptance by consumers but this could be overcome by their education and proper labelling of irradiated food [33].

### 4.2. High pressure

The idea of high pressure implemented as a food preservation method started at the end of the 19<sup>th</sup> century with the first studies, but it gained importance only during recent decades because of the rising demand for food with preserved nutritive properties [34]. High pressure processing (HPP) includes the use of a pressure chamber at 100-600 MPa, transmitted via water as the pressure transfer medium, and conducted at room temperature. The preservative effect is based on the destruction of microorganisms as well enzyme inactivation, while nutritive value is not affected. In fresh meat, pressure above 150 MPa starts to change the colour by affecting myoglobin, so PHH is more appropriate for cured meat products such as cooked and dry cured ham. Because of the differences in bacterial resistances to high

pressure, hurdle technology using a combination of other antimicrobial parameters could provide a stronger preservation effect at low pressure [35]. HPP could have special importance as a post-packaging processing step in the control of *Listeria monocytogenes* in ready-to-eat products. Furthermore, the fact that this technology is environmentally friendly and waste free could contribute to its greater importance in the future [34]. However, it is considered that the possibilities of HPP are still underestimated because of the lack of activity from large companies, and limited commercial research as well as consumer awareness of its benefits [36].

#### 4.3. Pulsed electric field

Pulsed electric field (PEF) is based on the discharge of high voltage short electric pulses that leads to permeabilization of cell membranes of microorganisms without negative impact on the nutritive value of food. It is mainly used for liquid foods and has no wide use in the meat industry [37], but some studies indicate some interesting approaches concerning the use of PEF (1-3 kV/cm in 100 pulses) as a support for drying, marinating and salting of meat. Specifically, PEF could increase both the speed of salt diffusion and drying during production of dry cured ham, provide faster fermentation in fermented sausages and improve brine distribution in cooked ham production [38].

#### 4.4. Pulsed light

Pulsed light (PL) is based on short time light pulses which are capable of inactivating microorganisms on food surfaces. The antimicrobial effect is provided through the UV spectra of the light, which damages the microbial DNA. PL treatment includes 1-20 light flashes per second, applying energy from 0.01 to 50 J/cm<sup>2</sup> [39]. There are several possible uses in the meat industry, such as decontamination of carcasses (skin and meat) [40], improved safety of fresh food products such as beef and tuna *carpaccio* [41], decontamination of equipment (knives) after being in contact with meat and meat products [42] and sliced fermented sausages [43]. As too intensively pulsed light could lead to changes in sensory properties of meat, especially colour and aroma, there are some limitations concerning the intensity of the treatment which should be optimised according to the number of flashes, voltage, spectral range and the distance between the product and the light source. Another concern is the economic aspect because of the need for specific equipment [39].

#### 4.5. Cold plasma

Cold plasma (CP) consists of an ionized gas that contains ions (+ and -), electrons, free radicals and photons. Such accumulated charged particles act destructively on microorganisms, providing the antimicrobial effect. CP could serve for decontamination of surfaces of equipment and meat and meat products [37], but as some adverse effects on fresh meat colour were observed, and as lipid oxidation and a certain degree of off-flavour developed, a balance must be found between the antimicrobial effect and sensory qualities of the product [44]. An interesting approach could be application of plasma-treated water (PTW) as a curing agent in emulsion type sausages, because plasma treatment of water generates NO<sub>2</sub><sup>-</sup> ions which could give colour to the sausages as if they were treated with nitrite [45].

### 5. Functional meat products

The concept of functional food has gained interest with consumers' awareness of the influence of nutrition on their health and includes the approach of food modification to reduce the amount of potential harmful components and enrich food with health promoting components. As meat products are often described as potentially harmful because of their saturated fat and salt contents, as well as the moieties formed through smoking and curing, most research in this area deals with these issues [4]. Saturated fat reduction is based on the replacement of animal fat with prebiotics, with promising results obtained with inulin [46,47] and with emulsion systems containing oils rich in polyunsaturated fatty acids (PUFAs) [48,49]. The possibilities of the reduction of N-nitrosamines, PAHs and sodium were discussed in other sections of this paper. As for enrichment of meat products with functional ingredients, probiotics could be successfully implemented in fermented sausages, prebiotics both in fermented and heat treated products simultaneously serving as animal fat replacers, minerals such as

salts of Ca, Mg or K could be used as partial NaCl replacers and at the same time, would serve as functional ingredients [23, 47]. The main problems concerning functional meat products enriched with PUFAs are their proneness to lipid oxidation as well as the adverse influence of the oils on sensory properties, but novel techniques in oil emulsification such as organogelation, oil bulking, structured emulsions and microencapsulation could help to overcome these issues [50].

## 6. Traditional meat products

The production of traditional meat products leans heavily on the oldest processing techniques, including salting, smoking, fermentation and drying. These products are highly appreciated by consumers and have a special value on the market. Although traditional production is, in general, considered as “safe”, there can be some safety issues concerning some parasites (*Trichinella* spp.), bacteria (*C. botulinum*), moulds and PAHs etc. Therefore, there is a need for food safety management systems for small producers, based on good hygiene practice and good manufacturing practice or some sort of generic hazard analysis and critical control points plan [9]. Small producers are not often capable of increasing production in order to meet growing market demands, whereas industrial companies try to take advantage of market desires. Industrial production, though, often leads to changes including lower quality of raw materials, use of starters, additives, artificial casings etc. Because of that, traditional principles should be implemented in industrial production of such products in order to achieve the expected product quality demanded by consumers [51].

## 7. Conclusions

Meat processing techniques continuously change following contemporary scientific and technological achievements. The oldest processing methods such as chilling, freezing, salting, smoking and drying still remain irreplaceable but have, nowadays, been modified with respect to equipment and consumers' demands about health issues. Novel methods, such as irradiation, high pressure, pulsed electric field, pulsed light and cold plasma still present some obstacles, including consumer acceptance, equipment costs and preservation of sensory properties of the meats. The functional food concept in meat processing struggles with oxidation issues and maintaining the required sensory properties of PUFA-enriched products, with promising results obtained by improving emulsification processes or utilising microencapsulation. Traditional meat products are evergreen in the area of meat processing, with growing demand for these products from consumers requiring introduction of flexible safety management principles, although the production principles and quality should be unchanged.

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# The lack of virus control in oysters could lead to a norovirus outbreak

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**Abstract.** Norovirus, a genus in the family *Caliciviridae*, is a leading cause of viral gastroenteritis in humans and responsible for many outbreaks worldwide. Filter-feeding shellfish are important vehicles for transmission of foodborne pathogens, including enteric viruses such as norovirus, when grown in sewage-polluted water. In this study, we investigated a norovirus outbreak linked with consumption of oysters and mussels. In January 2019, a gastroenteritis outbreak was identified involving eight patients with symptoms of gastroenteritis. Norovirus was diagnosed in stool samples using immunochromatographic test RIDA® QUICK Norovirus, and confirmed with real-time PCR. Of four shellfish (oysters, mussels) samples analyzed using real-time PCR, three were norovirus GI-positive and GII-positive, while one sample was only GII-positive. Six stool samples were collected, two of which were norovirus GI-positive and GII-positive, while three were GII-positive only. Following phylogenetic characterization of the human stool viruses, five out of eight belonged to the GII.6 cluster. Shellfish collected during this outbreak investigation contained the same GII.6 sequence. This is the first norovirus outbreak connected with shellfish consumption in Croatia. Understanding the transmission routes and vehicles of norovirus outbreaks is of great public health importance, and these results imply the co-circulation of GII.6 norovirus in people and oysters in Croatia.

## 1. Introduction

Foodborne viruses are an important and emerging problem for food safety and public health, according to a report by EFSA [9]. In 2014, viruses were, for the first time, the most commonly detected (20.4%) causative agent in foodborne outbreaks. Since the discovery and identification of Norwalk virus in 1972 [16], human noroviruses have been identified as the leading cause of acute viral gastroenteritis worldwide [25]. This genetically diverse group of viruses forms a separate genus in the family *Caliciviridae*, and the noroviruses cluster phylogenetically into at least seven genogroups with more than 30 genotypes located within three human-occurring genogroups (I, II, and IV) [47]. GI and GII viruses are responsible for the majority of disease in humans, whereas GIV viruses are rarely



detected as the cause of epidemic or sporadic disease [2,45]. It has been estimated that norovirus infections cause 1 million hospitalizations and 200,000 deaths in children under 5 years of age in the developing world [31].

The association between shellfish and viral gastroenteritis has been recognized for a long time [27]. Filter-feeding shellfish, when grown in sewage-polluted water, are important vehicles for transmission of foodborne pathogens, including enteric viruses such as norovirus and hepatitis A virus [23]. Numerous shellfish-associated outbreaks have been attributed to enteric viruses, particularly norovirus [39, 18, 34, 13]. The risks related to consumption of shellfish are greater when these products are eaten raw or lightly cooked, as in some European countries, including the Croatia.

European Regulation 2073/2005, and subsequent amendments, defines food safety criteria of shellfish only on the basis of bacterial indicators (e.g., *Salmonella* and *Escherichia coli*), which may not be correlated with the presence of viruses [7, 18].

Consequently, foodborne viruses are recognized among the top food safety priorities in a recent report by risk assessment experts who applied the Delphi technique [36]. Thus, over the past few years, foodborne viruses have become a greater concern to both the food industry and regulatory bodies [4]. In countries where rotavirus vaccines are implemented, norovirus has surpassed rotaviruses as the most common cause of childhood gastroenteritis requiring medical attention [32].

Gathering information on virus contamination in shellfish has, therefore, become increasingly important in countries with shellfish production. In 2017, Croatia produced 920 tonnes of *Mytilus galloprovincialis* and 62 tonnes of *Ostrea edulis*, according the Croatian competent authority [14].

## 2. Materials and methods

Epidemiological data: Information concerning sick oyster consumers was provided by medical doctors, who diagnosed norovirus gastroenteritis and informed the veterinary inspection services. When a foodborne illness is suspected, a standardized questionnaire concerning all consumed food is completed by the people who shared the meal. Following the implication of oysters in the outbreak, veterinary inspectors collected two oyster (*Ostrea edulis*) and two mussel (*Mytilus galloprovincialis*) samples from two producers that were directly linked to the human illness cases.

### 2.1. Environmental Data and Sampling

The shellfish growing area is in Maloston Bay, located between the southeast-northwest oriented coast and Pelješac Peninsula. The majority of oyster production in that area is consumed locally, so the main source of contamination is not identified. According to European regulation 54/2004/EC, the production area is classified as a Category A area. Oysters were collected from two producers at two production sites, of which one site is regularly surveilled for *Escherichia coli* levels. That site is considered to be representative of the microbiological contamination for that part of production area. Each shellfish sample comprised at least 15 individual oysters or mussels. On the first sampling day (1 February, 2019), four samples were collected and on the second sampling (20 February, 2019), ten samples were collected. All shellfish samples were transported in refrigerated boxes and were subjected to viral analysis for determination of norovirus GI and GII using the molecular method described by international standard EN ISO 15216-1:2017 (International Organization for Standardization).

### 2.2. Shellfish processing

All the shellfish were shucked, weighed, and their digestive tissues (DTs) dissected and homogenized. For analysis, Mengovirus (MgV) was added to each DT sample (2 g), before incubation with 2 ml of proteinase K solution (EN ISO 15216-1 2017).

### 2.3. Stool processing

Stool samples from patients with viral diarrhea were collected and transported to the laboratory for testing. RNA in the stools was extracted from 500 µL of a 10% stool suspension in phosphate buffered

saline (PBS, pH 7.4). One human stool sample was tested using a commercial immunochromatographic test RIDA® QUICK Norovirus (31 January, 2019).

#### 2.4. Nucleic Acid (NA) Extraction and Purification

Amounts of 2.0 g DT, spiked with 10 µl of process control MgV, were digested with 2 ml of proteinase K (0.1 mg/ml) at 37°C for 60 min with shaking, and then placed at 60°C for 15 min to inactivate the enzyme. Finally, the samples were centrifuged at 3000g for 5 min, and the supernatant was collected. Volumes (100 µl) of each stool sample was suspended in 1 ml of phosphate buffered saline (PBS, pH 7.4) and spiked with 10 µl of MgV. Nucleic acid extraction and purification were performed using the Nuclisens extraction kit (BioMerieux, Paris, France) according to the manufacturer's instructions, and the eluted RNA (100 µl) was stored at -80°C until real-time PCR (RT-PCR) analysis.

#### 2.5. RT-PCR and genotyping

RT-PCR for norovirus detection was carried out on a Mastercycler EP realplex (Eppendorf, Germany) using amplification conditions, primers and probes reported in EN ISO 15216-1:2017. RT-PCR reagents QuantiTect Virus + ROX Vial kit (Qiagen, Germany) were prepared according to the manufacturer's instructions. The presence of PCR inhibitors was evaluated by testing samples along with an external control RNA (EC-RNA of target sequence) and amplification efficiency.

All GII norovirus-positive samples detected by RT-PCR were further amplified by conventional nested RT-PCR, using the primer pairs COG2F/G2SKR and G2SKF/G2SKR that targets the partial capsid region C [15, 17]. The PCR amplicons were purified and subjected to direct sequencing on both strands (Macrogen). Typing of norovirus sequences was performed using the Norovirus Automated Genotyping Tool (<http://www.rivm.nl/mpf/norovirus/typingtool>). The phylogenetic analysis of aligned partial capsid sequences (300 nt) was carried out using MEGA 7. The reliability of the phylogenetic tree was assessed by bootstrap sampling of 1,000 replicates, and genetic distances were calculated by Tamura-3 parameter method.

### 3. Results

In January 2019, a gastroenteritis outbreak was identified and linked with consumption of oysters and mussels sourced from the Maloston Bay production area. Eight patients presented with symptoms of gastroenteritis (vomiting and/or diarrhea), and one patient was diagnosed as having norovirus by a medical doctor using immunochromatographic test RIDA® QUICK Norovirus on 31 January. The epidemiological investigation implicated oysters and mussels from two producers. Four shellfish samples were collected on 1 February and tested using RT-PCR. Three shellfish samples were positive to both norovirus GI and GII, and one shellfish sample was GII-positive. Stool samples from six patients were collected on 12 February, two samples of which were positive to both norovirus GI and GII, and three samples of which were GII-positive. Norovirus was not detected in one stool sample. On 20 February, ten more shellfish samples were tested, four of which were GII-positive (Table 1). The production area was closed until the confirmation of negative RT-PCR results on 5 March, 2019.

**Table 1.** Results of norovirus detection in shellfish and stool samples

Samples	Date	Quantity	Norovirus GI+	Norovirus GII+	Norovirus GI and GII+	Genotyped as GII.6
Oysters	01 February	2	2	2	2	1
Mussels	01 February	2	1	2	1	1
Stool	12 February	6	2	5	2	5
Oysters	20 February	5	0	1	0	0
Mussels	20 February	5	0	3	0	3
Oysters	05 March	5	0	0	0	0

Mussels

05 March

5

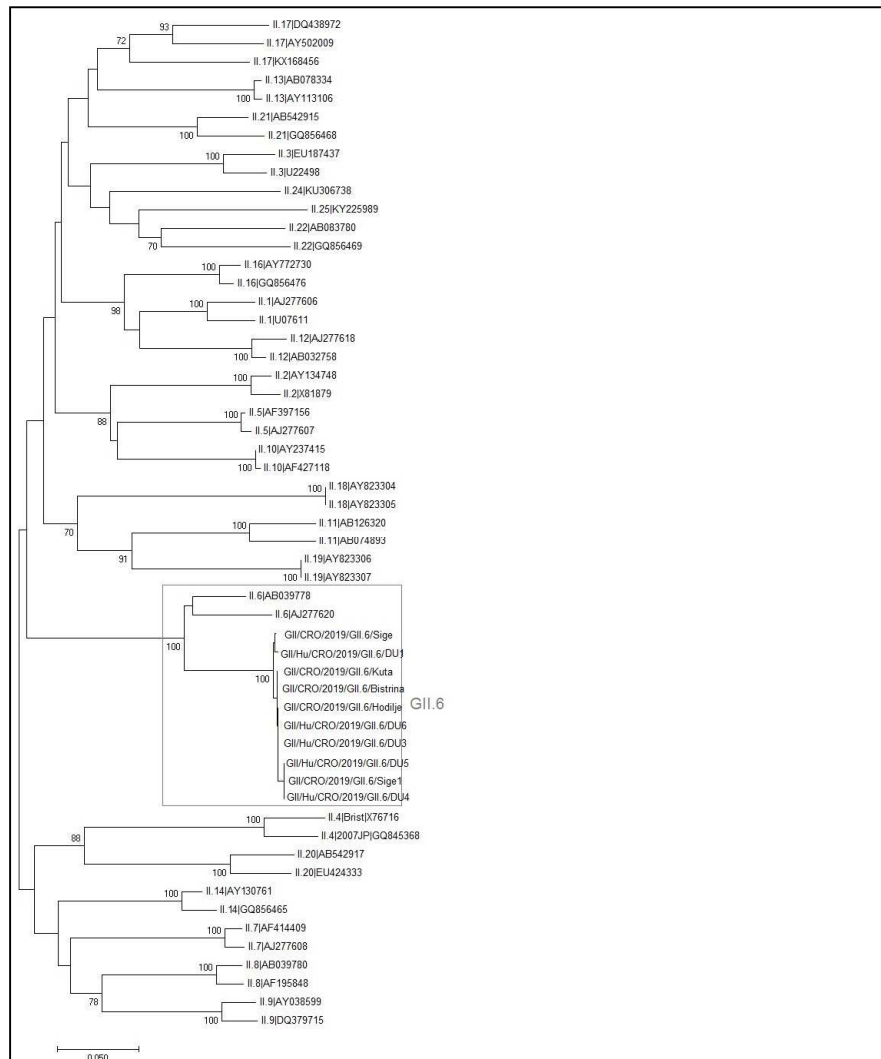
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Of the 11 GII-positive samples further amplified by nested RT-PCR, 10 were characterized by direct sequencing of the amplicons, identifying a single norovirus genotype, GII.6 (Figure 1). Phylogenetic analysis confirmed clustering of our human and shellfish sequences into a single cluster.



**Figure 1.** Neighbour-joining phylogenetic tree based on partial capsid viral protein 1 norovirus sequences. The GII.6 genotype is indicated with the square

#### 4. Discussion

The current European legislation classifies molluscan shellfish harvesting areas into A, B or C categories. Regulations based on measurement of the levels of *E. coli* in shellfish tissues (European regulation 54/2004/EC) or fecal coliforms in shellfish-growing waters (United States National Shellfish Sanitation Program) have been instituted to protect consumers. Viral contamination decreases very slowly within shellfish tissues, and although the use of depuration tanks is efficient in the case of bacterial contamination, it has little utility in addressing viral contamination [20,24,29,22].

In this study, we investigated a norovirus outbreak linked with consumption of oysters and mussels. One of the production sites implicated in the outbreak is considered to be representative for microbiological contamination, and the Maloston Bay production area is classified as A category.

Sanitary controls in Croatia are based on *Escherichia coli* counts in shellfish flesh and liquor as an indicator of fecal (sewage) contamination in shellfish [1], as in many other countries. However, this approach has been repeatedly demonstrated to inadequately indicate the risk from human enteric viruses [5,12]. In our case, patients who had consumed A category oysters and mussels developed gastroenteritis due to viral contamination. In one patient, norovirus was diagnosed using the immunochromatographic test, RIDA® QUICK. The outbreak investigation confirmed (using RT-PCR) the presence of norovirus GI and GII in oysters and mussels from two producers implicated in outbreak.

Human stool samples collected 12 days after the onset of symptoms tested norovirus GI-positive and/or GII-positive, using RT-PCR. According to Plantenga et al [33], norovirus can be readily confirmed as the cause of an outbreak of acute gastroenteritis if specimens are collected at any time during the first seven days after onset of diarrhea and (although the period is somewhat variable) for almost that long after symptoms resolve. No apparent decline in sensitivity occurs for specimens collected up to 6 days after onset of diarrhea. Sensitivity drops during days 7-14, but remains substantial for up to 2 weeks [33]. In our case, we positively detected norovirus in ill patients' stools collected on day 12, but probably with higher Ct values than would have been measured if we collected samples during gastroenteritis.

Genotyping of PCR-amplified partial capsid sequences obtained from human stools and from shellfish revealed a single norovirus genotype, GII.6. Phylogenetic analysis of shellfish and stool sequences confirmed the same epidemiological strain of GII.6 and provided a proven link of the outbreak with consumption of oysters and mussels. Sequences are difficult to obtain from oyster samples with low levels of norovirus [22] such as those implicated in this outbreak, and stools were collected only 12 days after the symptoms, not earlier. Despite these difficulties, we were still able to obtain partial capsid sequences. For many years, oysters have been known as concentrators of virus particles. Among shellfish, oysters are the most common vector of foodborne illness, and the pathogens most frequently involved in these outbreaks are noroviruses, responsible for acute gastroenteritis in humans [21]. The patients involved in this outbreak eat raw oysters and lightly cooked mussels. Consumption of either raw or undercooked shellfish can lead to transmission of disease, as human pathogens can be accumulated during the shellfishes' filter-feeding activity [38]. Several studies have examined the duration of norovirus excretion and found the average period of shedding is  $\approx 28$  days (range 13-56 days), well after the resolution of symptoms [44,3]. However, viral infectivity cannot be inferred from these findings. Considering the number of outbreaks linked to oyster consumption that have occurred in France, the French competent authority has developed a protocol that results in the growing area being closed for at least 28 days following an outbreak [22]. In our case, negative results were obtained on the 33<sup>rd</sup> day from the start of the outbreak, a temporal frame that is similar to the French experience. Consequently, the production area was closed for 33 days, since we did not have information about the status of the shellfish on the 28<sup>th</sup> day.

Overall, contamination by multiple norovirus strains has been reported in 65% of reported outbreaks, with GI and GII noroviruses detected, respectively, in 71% and 88% of stool samples and in 75% and 92% of shellfish samples [21]. In the current study, we also detected noroviruses GI and GII co-existing in some of our shellfish samples.

Shellfish are a high-risk food for viral outbreaks, but clear strain identification in shellfish is still often difficult [21], since obtaining a useful sequence from positive RT-PCR foods is problematic [37]. Specific binding of Norwalk virus in *Crassostrea gigas* oysters via a carbohydrate structure very similar to human histo-blood group A antigen was demonstrated and subsequently confirmed to occur in another oyster species (*Crassostrea virginica*) [43]. A simple depuration process should be sufficient for virus removal from oysters, as observed for bacteria [36]. However, long-term virus persistence in shellfish is a serious public health issue, and depuration or relaying is known to be inefficient [28,35,24]. Ligands that facilitate bioaccumulation (the A-like antigen) or that contribute to the elimination of the virus (the sialic acid-containing ligand) could both influence norovirus accumulation and survival in oysters [21].

One characteristic of shellfish-related outbreaks is their frequent association with multiple virus strains observed both in infected patients and in the involved shellfish [21]. In this study, we obtained one sequence of GII.6 in shellfish and stool samples. During the 2014 summer season in Croatia, a norovirus strain genetically related to Hu/GII.4/sydney/NSW05 in human samples, and simultaneously, GII.4 in shellfish originating from Croatian production areas were detected [40]. In humans, the genetic diversity of noroviruses is reflected in their binding capacity to various HBGA structures [42], and such differences also occur in oyster tissues [43,26]. When a number of different virus strains are detected in patients, association of the infection with shellfish consumption can be difficult if only a few stools from an outbreak are collected. Thus, it is essential to collect as many stool samples as possible from affected individuals, so that all strains that are present can be identified. It is also important to rapidly identify the outbreak in order to trace the oyster production and to quickly collect suitable samples related to the outbreak. These data can be used with collected epidemiological data to fully understand the role played by shellfish in the outbreak.

To assess risks associated with viruses and other hazards in the food chain and put in place appropriate control measures, the use of risk assessment techniques has been suggested by international bodies [6,46] and increasingly accepted by governments around the world as a basis for national legislation in relation to food safety [11,8]. Implementing raw material/food production controls (oysters, berries, leafy greens) e.g. harvesting oysters and other shellfish from non-contaminated areas, establishing an acceptable limit for norovirus in oysters to be harvested and placed on the market, and testing of products for compliance to this acceptable limit [9] are examples of these controls. The periodic emergence of viral outbreaks associated with shellfish consumption continues to pose a real public health concern. Although outbreaks related to consumption of shellfish in Croatia have not been previously reported, it is well known that human sewage is a possible source of shellfish contamination. This is the first reported case of a norovirus outbreak related to shellfish consumption in Croatia. Understanding the transmission routes and vehicles of infection for norovirus outbreaks is of great public health importance, and suitable control measures for viruses should be implemented, especially for oysters since these are eaten raw.

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# Noroviruses in shellfish: Challenges and facts

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**Abstract.** Noroviruses are among the most common causes of foodborne outbreaks of human infection. Study of the noroviruses has been difficult due to the lack of a cell culture system, so molecular techniques became the gold standard. Recently though, a cell culture system has been developed and will aid in the acquisition of new knowledge related to human noroviruses.

## 1. Introduction

Noroviruses cause large gastroenteritis outbreaks worldwide (1,2). They are highly contagious because of their low infective dose, their inducement of short-term immunity and their stability in the environment (3). The most common route of infection is person-to-person transmission through the faecal-oral route or by exposure *via* contaminated surfaces (4). Foodborne transmission can occur *via* contamination of food by infected food handlers or directly from contaminated foods. Asymptomatic norovirus infections are also common (5,6,7). Foods that are often implicated in norovirus outbreaks are leafy greens, fruits (raspberries), and shellfish (8,9,10).

## 2. Food safety regulation

Shellfish harvesting areas are classified under EC Regulation 854/2004. According to the degree of faecal pollution based on *Escherichia coli* levels, harvesting areas are categorized as A, B or C category. Shellfish from A category are sold directly on the market, whereas shellfish from category B and C undergo a purification or relaying process. Regulation 2073/2005 prescribes criteria for *E. coli* but not for viruses; *E. coli* in the sampled shellfish should not exceed 230 MPN/100 g in 4 out of 5 samples and no more than 700 MPN/100 g in one sample of shellfish flesh. Numerous studies agree on the resistance of human enteric viruses in shellfish during the depuration process, which is, on the other hand, effective for bacterial elimination (14,15,16). The combination of a relaying period with a final purification process allowed the diminution of noroviruses to low copy numbers (17). Cooking at 90 °C for 90 seconds is enough to inactivate enteric viruses (18).

## 3. Detection of the noroviruses

Before ISO 15216-1:2017 for determination of norovirus (and hepatitis A virus) was released, many different methods for the detection of noroviruses were used. The ISO describes methods of norovirus detection for food surfaces (swabs), soft fruit, leaf, stem and bulb vegetables, for bottled water and for shellfish (11). As noroviruses accumulate in the digestive glands of the shellfish (12), the extraction is made from the digestive glands using treatment with a proteinase K solution (11). Detection is made using real-time reverse transcriptase-PCR (RT-PCR) with different primers and probes.



Detection of noroviruses in shellfish samples is difficult because of the low contamination level, the presence of substances that inhibit molecular detection, wide genetic variability and the difficulty of efficient virus extraction (13).

### 3.1. Classification of the noroviruses

Noroviruses are nonenveloped positive-sense single-stranded RNA viruses, classified in the family *Caliciviridae*. Noroviruses are classified into genogroups and genotypes based on amino acid diversity in the complete VP1 protein (19). Seven genogroups have been classified, and each genogroup is further divided into genotypes. Genogroup GI, GII, and GIV are human noroviruses (19), GII is the most commonly detected in clinical cases, whereas GIV is rarely detected (20). Most of the reported quantitative norovirus RT-qPCR assays target the ORF1-ORF2 junction region, because this part of the norovirus genome is sufficiently conserved for the development of genogroup-specific oligonucleotide primers and probes.

### 3.2. Pandemic outbreaks

Among all genotypes, only GII.4 is associated with pandemics. Since the mid-1990s, outbreaks and sporadic cases of genogroup II genotype 4 (GII.4) have frequently been reported (6, 21) and have caused five pandemics of acute gastroenteritis (22). Variants of GII.4 have emerged every 2 to 3 years (19). The predominant GII.4 strains had a higher mutation rate and rate of evolution compared to the less frequently detected strains. The GII.4 lineage had higher rate of evolution within the capsid sequence compared to other noroviruses. The study of Bull et al., 2010 supports the hypothesis that epidemiological fitness is a consequence of the ability of the virus to generate genetic diversity, as the pandemic GII.4 strains were associated with increased replication and mutation rates. A parallel can be seen in the epidemiology between norovirus and influenza virus (22). In 2014, a new GII.17 variant known as Kawasaki 2014 emerged and caused an outbreak and sporadic cases across the world, and is replacing the previously prevalent GII.4 Sydney strain from 2012 (23,24,25). In the study of the norovirus strains isolated from sewage in Japan, a strain closely related to the GII.17 Kawasaki 2014 lineage had been observed in the study area a year before its appearance in the clinical cases. A similar pattern was also observed for GI.3 (26).

In a meta-analysis of norovirus global seasonality, winter peaks of noroviruses and positive association with average rainfall in the wettest months were shown (27). The number of gastroenteritis cases increased in the winter months, from November to February, and decreased in the summer months, from June to August. These trends were similarly observed in each norovirus season (26). In Slovenia, a study of norovirus strains isolated from mussels harvested at three harvesting areas (Seča, Strunjan and Debeli rtič) has been conducted. Sequence similarity among strains detected in mussels, strains isolated from sources of drinking and surface water and strains from human clinical samples was 95% at the nucleotide level and 100% at the amino acid level. A sequence similarity study showed up to 100% match at the nucleotide level with other human strains isolated worldwide (Japan, China, Korea, India). Higher levels of contamination at the Debeli rtič harvesting area (25.9% of mussels contain norovirus), the most northern point where the main Adriatic Sea current flows, can be attributed to intensive shipping in this area neighbouring the port of Koper, discharges of waste-waters, the river estuary and the main sea current that flows north (28).

## 4. Cultivation of noroviruses

Until recently, there were no available cultivation system for human noroviruses, so the discovery of Ettayebi and collaborators was revolutionary (29). Stem-cell-derived intestinal enteroids from duodenum, jejunum, or ileum were used as *in vitro* culture systems for human noroviruses. These cells are susceptible to human norovirus infection and exhibit cytopathic effects. Special protein staining revealed that specifically enterocytes were infected. It was shown that human norovirus infection is dependent on bile acids in enteroid cultures (29).

Recent studies furthermore indicate that infection by noroviruses is also influenced by the commensal microbiota (30,31). B cells have been identified as a cellular target of noroviruses, whereas enteric bacteria could serve as a stimulator for the infection. Human norovirus infection of B cells required

the presence of free HBGA or HBGA-expressing bacteria, for example *Enterobacter cloacae*. When the intestinal microbiota was depleted by oral antibiotic administration, norovirus replication was reduced *in vivo* (30). On the other hand another bacterium, *Lactobacillus*, helped the recovery of human intestinal microbiota after norovirus infection (32).

## 5. Conclusion

There has been great progress in the field of detection and cultivation of the noroviruses in recent years. With the development of the cell culture system and the discovery of some cofactors critical for the replication of noroviruses there are some new answers, but new questions are arising. Noroviruses are a heterogeneous group of viruses, with a variety of strains and new variants. The gold standard for the detection is RT-qPCR, but new technologies, next generation sequencing and whole genome sequencing are promising tools for a broader spectrum of information about the whole norovirus genome.

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# Food safety aspects of common carp produced in wastewater-fed fish ponds

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**Abstract.** There is an increasing trend in the use of wastewater in fish production because wastewater-based aquaculture is a sustainable, biological way to treat and recycle wastewater. Different fish species including common carp have been reared in wastewater-fed ponds. However, untreated wastewater contains many kinds of contaminants that have adverse effects on human health and the environment. Thus, the health risks arising from fish produced in wastewater-filled fish ponds seem undeniable. On the other hand, the appropriate use of wastewater in aquaculture has important environmental and economic significance, including recycling nutrients and reuse of water. The main objective of the present review is verification of rearing fish in purified wastewater-fed fishponds through revision of available data related to fish meat safety. Wastewater could be an excellent source of nutrients for fish, but proper purification of this waste stream is necessary. Fish also have a role as bioindicators of the effectiveness of wastewater purification. Continuous monitoring of the presence and concentration of different contaminants in common carp and other fish species reared in purified wastewater is very important.

## 1. Introduction

The use of wastewater in aquaculture is a common practice in some parts of the world, mainly in Asian countries, but there are increasing possibilities for such practice in Europe and in Serbia. Moreover, different integrated food production systems involving aquaculture are used in Asian countries [1]. Such systems contribute to optimal use of land, water and solar energy in order to reach high yields with low financial investment. Wastewater provides a good source of nutrients for fish and consequently reduces the need for fertilisation of fish ponds. Besides, these advantages, various integrated livestock-fish culture systems utilise animal excreta as fish pond fertilisers. Such practice contributes to growth of plankton and other organisms in the ponds and consequently to fish growth. Thus, wastewater-based aquaculture is a sustainable, biological way to treat and recycle wastewater. The main objective of this review is verification of rearing fish in purified wastewater fed fishponds through revision of available data on fish meat safety.

Different fish species have been reared in wastewater-fed ponds. Filter feeders are recommended due to their ability to exploit plankton, but omnivorous species and bottom feeders such as common carp are also considered suitable for this purpose [2]. Common carp is one of the most commonly reared fish in different parts of the world [3]. Fast growth rate, beneficial feed conversion ratio and relative resistance to poor environmental conditions and diseases are the main reasons that common carp is a highly esteemed fish species, and that it is the most widespread farmed fish species in Serbia. Moreover, these characteristics contribute to the fact that common carp could be successfully reared in wastewater-fed



ponds or various types of integrated culture systems. The main reason for increasing demand for fish including common carp is that fish is considered as a healthy food [4]. Moreover, fish is an excellent source of fatty acids in healthy nutrition [5]. On the other hand, consumers are increasingly directing their attention towards safety requirements associated with fish consumption due to the presence of different environmental contaminants, especially if fish are reared in wastewater-fed ponds or integrated production systems. It is important to increase public awareness related to the risks associated with consumption of fish containing these contaminants.

## **2. Use of wastewater in aquaculture**

There is increasing use of wastewater in fish production, particularly in developing countries. The alternative aquaculture production systems such as utilising wastewater for fish rearing or different integrated fish production systems are important from various viewpoints: environmental protection, sustainability, food safety, food quality, economic significance and scientific importance. However, there are still many questions regarding the use of wastewater in aquaculture. This topic is also very important from the human health protection point of view, since many industrial facilities discharge wastewater directly into waterways, which can compromise environmental quality. On the other hand, purified wastewater could be an excellent source of nutrients for fish. Wastewater used for this purpose must be purified as a necessary first step. Fish also have a role as bioindicators of the effectiveness of wastewater purification.

## **3. Public health risks related to the use of wastewater in aquaculture**

Rearing fish in wastewater could produce significant risks for public health [6]. Besides, fish can act as bioindicators of environmental contamination. Thus, the safety of fish reared in wastewater-filled ponds is a public health concern. The potential public health risks associated with this practice include bacterial and parasitic infections like diarrhoea and skin infections. Untreated wastewater can contain many kinds of contaminants with adverse effects on human health and the environment such as pathogenic microorganisms, heavy metals, pesticides, antibiotics and hormones [7]. On the other hand, the appropriate use of wastewater in aquaculture has important environmental and economic significance, including recycling nutrients and reuse of water.

Dang and Dalsgaard [8] showed low levels of faecal contamination in muscle tissues of silver carp, grass carp and rohu reared in household-based integrated systems where pig farming was integrated with fish farming and horticulture. However, high levels of *Escherichia coli* were observed in the gut of the studied fish. The authors concluded the prevention of faecal cross-contamination during degutting and preparing fish for consumption at the market or in the home are the main critical points to control the food safety of fish flesh produced in integrated systems. Edwards [9] noted high levels of *E. coli* in the fish digestive tract contents but low levels in the muscle tissue of fish from fish ponds fed with urban wastewater in different Asian countries. Furthermore, fish reared in fish ponds fertilised with urban wastewater contained very low levels of thermotolerant coliforms in muscle tissue, but their skin and digestive tract contents were highly contaminated [10]. Development and transmission of antibiotic resistant bacteria and transmission of resistance genes to fish and humans are serious potential food safety risks related to integrated livestock and wastewater-based fish farming systems [11]. Antibiotic resistance can increase among microorganisms in aquaculture environments due to the selective pressure of the antibiotics used as growth promoters or for medical purposes [12]. Additionally, antibiotic resistant microorganisms could be introduced to wastewater-fed fish farm waters *via* animal manure.

Other food safety hazards associated with usage of wastewater in aquaculture production include fishborne zoonotic parasites that can be transmitted to humans through consumption of raw or improperly prepared fish [13]. Hop et al. [14] reported that fish from peri-urban wastewater-fed aquaculture systems are at risk of infection with trematodes, but the prevalence was low compared to previous findings of trematodes in non-wastewater fish elsewhere in Vietnam. Pigs can be a reservoir host for trematodes, and eggs could be introduced into fish ponds *via* infected pigs' waste [15]. The use of exclusively commercial feed in pig nutrition and proper heat treatment of fish before consumption are the main methods for prevention of transmission of trematodes. Furthermore, significant safety concerns in fish are toxic metals and metalloids that accumulate in fish after dietary exposure or

absorption through gills [16]. Unacceptable levels of arsenic, cadmium, mercury and lead in fish are serious food safety risks. The data regarding metal levels in fish reared in wastewater-fed ponds are very scarce. According to results obtained by Marcussen et al. [17], consumption of common carp, silver carp and tilapia flesh produced in wastewater-fed ponds in Vietnam did not present a food safety problem in terms of arsenic, cadmium and lead. On the other hand, Mansour and Sidky [18] reported that levels of cadmium and lead in fish caught in a lake receiving water from agriculture and fish production were above the threshold values [19]. Potentially toxic elements, mainly heavy metals, in fish and water spinach from Hanoi and Cheung Ek Lake in Phnom Penh constituted low food safety risks for consumers, and consumption of muscle tissue from fish produced in wastewater-fed systems resulted in an estimated intake of these elements amounting to less than 9% of the tolerable daily intake [20].

Pesticides are present in aquatic sediments and can be transferred into the aquatic environment and finally enter into the food chain [21]. Because their solubility in lipids is high, pesticides tend to accumulate in fatty tissues of fish [22]. According to WHO [23], some of the older, low cost pesticides, including dichlorodiphenyltrichloroethane (DDT) and lindane, can remain in soil and water for years. These pesticides are officially prohibited from use in agricultural practice in developed countries, but they are still in use in many developing countries, so they could be present in wastewater and consequently in fish from wastewater-fed fishponds. There is evidence of high concentrations of DDT and its metabolites in the environment in different parts of the world [24]. Having in mind all the above-mentioned risks associated with the use of wastewater, the purification of wastewater before use in aquaculture is necessary. Fish could have a role as bioindicators of the effectiveness of wastewater purification treatment. Khalil and Hussein [25] reported that the primary and secondary treated waste effluents were successfully used to grow Nile tilapia.

#### **4. Possibility of wastewater use in aquaculture in Serbia**

In Serbia, vast unused land areas near slaughterhouses or various food industry facilities are not cultivated or suitable for other agricultural activities but could be used for aquaculture. Currently, fish production in Serbia mostly consists of the traditional rearing system, which is a semi-intensive culture system, and the diet of fish is based on a combination of natural food and supplementary feed (cereals, such as wheat, maize, barley or extruded feed mixtures). Similar fish-farming techniques are found in many countries worldwide. Rural areas would be ideal hotspots for developing integrated fish production since the land is far away from human settlements, purified wastewater could be used for filling ponds, thus significantly reducing the release of harmful agents into waterways and the environment, and applying appropriate piscicultural practices could lead to favourable natural food composition. Wastewaters from slaughterhouses in developing countries are discharged into rivers, lakes and seas without being adequately treated. Such wastewater contains suitable amounts of organic matter that would be an ideal material for fish nutrition and for development of different microorganisms.

#### **5. Consumer attitude towards fish produced in wastewater**

The reuse of wastewater in aquaculture is accepted and practiced worldwide. However, it is very important that the whole process is applied with precautionary measures and strictly controlled. The presence of microbial pathogens, parasites and toxic chemicals in fish, water and sediment must be monitored. Also, the acceptance of such fish by consumers is a very important issue. According to Mancy et al. [26], consumers in Egypt did not accept fish produced in treated sewage water despite the fact that the fish produced were suitable for human consumption. Consumer behaviour and cultural habits could be the leading reasons against the use of wastewater in fish farming, besides public health and food safety concerns. Consumer acceptance of wastewater-based fish farming and the requirements of public health protection demand proper treatment of wastewater before its usage in aquaculture production.

#### **6. Preventive measures against harmful agents in fish**

The best preventive measures against microbial contaminants in fish are avoiding consumption of raw and uncooked fish. Health education is an important factor in minimising risks associated with

consumption of fish reared in wastewater-fed fishponds. Aquaculturalists must ensure that fish are subjected to visual inspection for parasites before being placed on the market [27]. There are also various obligatory microbiological and chemical analyses before fish can be placed on the market. Recommended methods for eliminating or minimising microbial contamination of fish are freezing, heating, combinations of salt and storage time and hot smoking among others [28]. The health risk is significantly reduced by adequate heat treatment of fish before consumption.

Most pathogens that enter wastewater-fed fish ponds migrate into and populate the pond sediment and so are health risks for anybody entering the pond during fish harvesting. Fish harvesters must adopt adequate personal hygiene measures for their own health protection. Guidelines for the safe use of wastewater and excreta in aquaculture [29] focus on microbial safety, but there are also some recommendations related to toxic elements. Depuration, i.e. holding fish in clean water for several weeks before consumption, is mentioned as a method for decontamination of fish reared in wastewater-fed ponds, but there is a need for more scientific evidence of this. The management of wastewater is still a great problem in the majority of countries worldwide and inadequate management could lead to serious health problems. The safe use of wastewater for fish rearing should be encouraged. For that purpose, proper treatment of wastewater must be applied before its use.

## 7. Conclusions

People employed at fish pond facilities, people living near these facilities and consumers of the fish produced are specific populations at risk of exposure to various contaminants related to integrated fish production systems and wastewater-fed fish ponds. Additional studies are required in order to understand the health risks associated with these types of fish production and to develop suitable measures for reduction or prevention of human health risks. Continuous monitoring of the presence and concentration of different contaminants in common carp and other fish species reared in purified wastewater is very important, having in mind that fish is an important food source but also is also an important indicator of environmental contamination. Research should provide important data for the exposure assessment part of risk assessments for contaminants from fish reared in purified wastewater. Furthermore, research would be helpful to fish processors for developing quality assurance programs for fish raised in integrated production systems. Undoubtedly, adequate treatment of wastewater is required before its use in aquaculture.

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# Modified atmosphere packaging of fish – an impact on shelf life

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**Abstract.** The shelf life of fresh fish is short. Therefore, the fishing industry has always been willing to explore new technologies for shelf life extension. This paper will focus on one such technique, i.e., changing the nature of the gas surrounding the fish. The main purposes of modified atmosphere packaging of fish and fish products, but also other foodstuffs, are two-fold: to ensure the microbiological shelf life and the sensory quality of the product, including the colour, odour and palatability. During the last two decades, modified atmosphere packaging has become a dominant retail fish packaging technology. The main reasons for the development of modified atmosphere packaging are the continuous increase in fresh fish consumption, increases of urban populations and exhaustion of natural food resources. Developments in packaging materials and technologies have made the application of modified atmosphere packaging on a larger scale for fish and fish products feasible. In this paper, we present the basic principles of modified atmosphere packaging of fish, and the microbiological, sensory, chemical and physicochemical parameters that are important for the shelf life of fish packaged using this technology.

## 1. Introduction

Proper nutrition is of primary importance for the quality of people's lives. Hence, fish meat, due to its nutritional value, plays a major role in human nutrition. An increasing number of people are aware of the nutritional value of fish, especially given that fish meat is significantly less burdened with the various additives that are used in modern pig and poultry farming. What makes fish particularly attractive to consumers is that, in addition to favourable contents of proteins, minerals and vitamins, it is a very rich source of essential fatty acids that play an important role in the prevention of numerous human diseases. Due to such properties, fish meat is one of the nutritionally most valuable foods.

The fact that fresh fish is a very perishable food (pH > 6.0; aw > 0.98) has induced producers to focus on finding more optimal methods for fish preservation. In recent years, however, consumers worldwide are increasingly demanding that they have fresh fish at all times, since this fish type has the most acceptable sensory properties. This trend has led to the development of modified atmosphere packaging (MAP) for fish and fish products, thus ensuring fish's longer shelf life and preservation of the basic parameters associated with fish freshness [1]. The shelf life of a foodstuff, and therefore fresh fish, can be defined as the time after food packaging, during which the product is primarily safe for consumption and during which the sensory properties of the product (colour, odour, flavour, texture) and its nutritional value remain unchanged and acceptable to the consumer.

MAP is used today in the production of fresh and chilled food, including raw and thermally processed meat, poultry, fish, pastries, fruits, and vegetables and, more recently, coffee, tea, and bakery products.



Market demand today, however, requires that the food should be processed to a minimum extent and free of additional preservatives and additives, so MAP food is increasingly present at retail.

## 2. Principle of MAP

MAP can be defined as removing air from the packaging and replacing it with a particular gas or mixture of gases. The purpose of this technology is to extend the shelf life of food by preventing or slowing down both the biochemical processes (lipid oxidation, reactions caused by activities of the fish's own enzymes) and the development of bacteria that lead to product spoilage. The most commonly used gases in MAP technology are carbon dioxide (CO<sub>2</sub>), oxygen (O<sub>2</sub>), and nitrogen (N<sub>2</sub>). These gases are used in different combinations, and their roles in the modified atmosphere differ. While N<sub>2</sub> is an inert and tasteless gas with the task of preventing packaging collapse, CO<sub>2</sub> inhibits the growth of several types of microorganisms, especially those that cause deterioration and unpleasant odours in foods stored at refrigerator temperatures. A major advantage of CO<sub>2</sub> is that it is not toxic to humans. CO<sub>2</sub> is highly soluble in water and fat and its solubility increases with decreased temperature. Therefore, the effectiveness of this gas is always conditioned by the food storage temperature, with increased inhibition of bacterial growth as the temperature is decreased. The bacteriostatic effect of CO<sub>2</sub> depends on its concentration and the temperature at which the food is stored, while the mechanisms of action are based on changes in the permeability of the bacterial cell membrane, inhibition of enzymes, change in the physico-chemical properties of the proteins, and change in the bacterial cell pH [2].

O<sub>2</sub> plays an important role in MAP, especially in fresh meat packaging. High levels of O<sub>2</sub> are used in MAP for red meat and red fish meat (tuna, yellowtail) to maintain the myoglobin pigment in the meat in an oxygenated form, thus giving the meat a bright red colour, acceptable to the consumer. However, the content of O<sub>2</sub> in MAP is normally kept as low as possible to inhibit the growth of aerobic spoilage bacteria.

## 3. Modified atmosphere packaging of fish and fish product

In the past decade, the attention of researchers engaged in solving problems associated with fish packaging has mostly been focused on gas mixtures with high concentrations of CO<sub>2</sub> and N<sub>2</sub>. The impact of MAP on fresh fish shelf life and the most appropriate mixture of gases, however, depend on the fish species to be packaged, the fat content, the initial microbiological contamination level, fish manipulation following the catch, the volume ratio of gas to fish in the package and, most importantly, the method of packaging and the storage conditions [3]. In some cases, MAP can adversely affect the quality of the packed fish due to CO<sub>2</sub> dissolution in the fish meat, resulting in the formation of carbonic acid. Moreover, at lower pHs, the capacity of fish meat to bind water is reduced, resulting in the separation of fish meat juice from fish in the package, and this juice is an ideal substrate for the development of spoilage microorganisms [3]. It is for these reasons that the optimum ratio of gases in the mixture must be determined. The ratio between the volume of gas and volume of food product (G/P ratio) should usually be 2:1 or 3:1 (volume of gas two or three times the volume of food). This high G/P ratio is also necessary to prevent package collapse, because the CO<sub>2</sub> solubility in the food means the gas volume reduces throughout the shelf life.

The shelf life of fresh, chilled fish is relatively short and at temperatures of 2±2 °C it is about 2 to 3 days. It has been confirmed that the packaging of fish in modified atmosphere significantly extends the shelf life of the product. The effect of MAP on the shelf life of foods in general has been reviewed by several authors in recent decades. In 100 % CO<sub>2</sub> atmosphere, fish kept fresh two to three times longer than control fish in air at the same temperature [4]. Fresh haddock, cod, sole, whiting and plaice were very effectively preserved under 20-100 % CO<sub>2</sub> atmospheres, with optimal conditions under 40-50 % CO<sub>2</sub> [5]. Since these early investigations, numerous research papers have been published on this topic, some reporting a tremendous increase in shelf life, others reporting little or no shelf life extension, but more often, a shelf life extension in the range of 30-60 % for fresh fishery products using atmospheres with high levels of CO<sub>2</sub> is observed. Table 1 summarises some of the more recent published articles concerning MAP and fish.

**Table 1.** Shelf life of fresh fish and fish products packaged under MAP, vacuum or air

Type of fish product	Storage temperature (°C)	Atmosphere CO <sub>2</sub> :N <sub>2</sub> :O <sub>2</sub>	G/P ratio	Shelf life (days)	Reference
Cod fillets ( <i>G. morhua</i> )	0	2:98:0	2	14	Dalgaard <i>et al.</i> [6]
	0	3:97:0	2	13	
	0	29:71:0	2	16	
	0	97:3:0	2	15	
Chub mackerel ( <i>Scomber japonicus</i> )	2	70:30:0	2	17	Goulas & Kontominas [7]
	2	50:30:20	2	11	
	2	vacuum	-	11	
	2	air	-	8	
Salmon slices ( <i>Salmo salar</i> )	2	100:0:0	ns	18	Pastoriza <i>et al.</i> [8]
	2	air	-	8	
Swordfish steaks ( <i>Xiphias gladius</i> )	4	40:30:30	ns	11-12	Pantazia <i>et al.</i> [9]
	4	vacuum	-	9	
	4	air	-	7	
Sea bass ( <i>Dicentrarchus labrax</i> ) fillets	4	60:40:0	3	14	Provincial <i>et al.</i> [10]
	4	50:50:0	3	14	
	4	40:60:0	3	11	
	4	air	-	4	
Sardine fillets ( <i>Sardina pilchardus</i> )	3	50:50:0	ns	9	Stamatis & Arkoudelos [11]
	3	vacuum	-	7	
	3	air	-	5	
Tilapia ( <i>Oreochromis niloticus</i> ) fillets	4	60:30:10	ns	15	Masniyom <i>et al.</i> [12]
	4	vacuum	-	12	
	4	air	-	6	
Carp steaks ( <i>Cyprinus carpio</i> )	3	60:40:0	2	12	Babić <i>et al.</i> [13]
	3	40:60:0	2	9	
	3	vacuum	-	7	
Carp steaks ( <i>Cyprinus carpio</i> )	3	100:0:0	2	15	Babić <i>et al.</i> [14]
	3	40:60:0	2	9	
	3	20:0:80	2	15	
Carp steaks ( <i>Cyprinus carpio</i> )	3	90:10:0	2	17	Babić Milijašević <i>et al.</i> [15]
	3	air	-	17	

ns: not stated in published paper

#### 4. Microbial parameters of importance for shelf life of fish packaged in MAP

There is no doubt the composition of the dominant microbiota of MAP fish and fish products depends on the mixture of gases used in the packaging. Microorganisms that otherwise cause meat spoilage in aerobic conditions are inhibited in MAP conditions by CO<sub>2</sub>, and hence their role in fish spoilage in the mixture of MAP gases is insignificant. It is for this reason that the predominant microbiota in fish packaged in MAP is CO<sub>2</sub> resistant. Unlike vacuum packing of fish, where conditions are such as to stimulate the growth of microorganisms that can use trimethylamine oxide (TMAO) as an O<sub>2</sub> source instead of O<sub>2</sub>, the mixture of MAP gases inhibits the growth of both trimethylamine (TMA)-producing microorganisms and the hydrogen sulphide producers. Basically, Gram-negative bacteria are much more sensitive to the inhibitory effect of CO<sub>2</sub>. This was confirmed in research with carp cuts packaged in different gas mixtures (100% CO<sub>2</sub> and 60% N<sub>2</sub>+40% CO<sub>2</sub>) and stored at 3 °C; numbers of mesophilic bacteria were lower in the carp packed in MAP with 100% CO<sub>2</sub> than in carp packed in MAP with 60%

CO<sub>2</sub> [16]. Significant reductions in the total number of mesophilic bacteria in fresh sea bass fillets, sardines and trout fillets packaged in MAP gas mixtures with high CO<sub>2</sub> contents were also reported [10,11,17].

According to International Commission on Microbiological Specifications for Foods (ICMSF) recommendations [18], the total number of mesophilic bacteria in fresh fish should not exceed 7 log cfu/g. A good correlation between sensory attributes and number of mesophilic bacteria in carp steaks packaged in MAP was reported by Babić [19]. At the moment when fish was assessed as unacceptable from the sensory point of view, the number of mesophilic bacteria was higher than this recommended limit (7 log cfu/g). The total number of mesophilic bacteria is a good indicator of overall acceptability of salmon fillets packaged in MAP with 40% CO<sub>2</sub> + 60% N<sub>2</sub> and stored at 0 °C [20]. However, in fresh sardines packaged in a MAP gas mixture (60% CO<sub>2</sub>+40% N<sub>2</sub>), vacuum and air, there was a poor correlation between overall acceptability and the total number of mesophilic bacteria, since the total number of bacteria reached the maximum recommended limit while the sensory attributes of sardines were still acceptable [21].

*Photobacterium phosphoreum*, resistant to CO<sub>2</sub>, is most commonly responsible for spoilage of MAP fish. The Gram-positive bacteria, such as lactic acid bacteria, primarily *Lactobacillus* spp. and *Leuconostoc* spp. as well as *Brochothrix thermosphacta* are not sensitive to the effect of CO<sub>2</sub>, and hence, these become the dominant genera in fish and fish products packaged in MAP gas mixtures. This has positive effects on fish shelf life, as these microorganisms have less potential to cause spoilage than some others [10]. It should be noted that the low level of carbohydrates in fish meat prevents the occurrence of the sour taste that results from the activity of lactic acid bacteria in the carbohydrate decomposition process. Generally speaking, the initial number of lactic acid bacteria in the fish meat is low and these bacteria are rarely responsible for fish spoilage. However, when growth of aerobic bacteria is inhibited, either by applying low temperatures or by modifying the atmosphere, lactic acid bacteria become the dominant bacterial population, as corroborated by the results of studies on carp cuts [22] and eviscerated trout [23].

During storage at -2°C of Atlantic mackerel fillets packaged in MAP with 100% CO<sub>2</sub>, growth in the number of lactic acid bacteria was determined; towards the end of the study, these become the predominant microbiota [24]. The results of numerous studies have shown that high concentrations of CO<sub>2</sub> (70-100%) result in the development of predominantly heterofermentative *Lactobacillus* spp. in MAP fish. Also, lactic acid bacteria constitute 62-85% of the microbiota involved in the spoilage of fish packaged in mixtures with higher concentrations of CO<sub>2</sub> (90-100%) [25].

## 5. Chemical and physico-chemical parameters of importance for shelf life of fish packaged in MAP

### 5.1. Total volatile basic nitrogen (TVB-N)

TVB-N is a chemical indicator of fish freshness. The total volatile N<sub>2</sub> is made up of compounds responsible for the formation of unpleasant odour and flavour in fish meat, among them being ammonia, dimethylamine (DMA), TMA, amines derived from decarboxylation of amino acids, and other nitrogenous compounds that become volatile when converted to their alkaline forms. Ammonia is formed in the process of bacterial disintegration of proteins, peptides and amino acids, as well as in autolytic decomposition of adenosine monophosphate (AMP). Dimethylamine and TMA are produced by the degradation of TMAO, a compound that plays a significant role in osmoregulation and the presence of which has been demonstrated in all marine and in a large number of freshwater fish species. The activity of endogenous fish enzymes results in the decomposition of TMAO and the formation of DMA and formaldehyde. In anaerobic conditions, and using TMAO as the ultimate electron acceptor in anaerobic respiration, the bacteria that cause fish spoilage foster the formation of TMA, a compound responsible for the characteristic odour of spoiled fish [26].

Packaging fish in a modified atmosphere is a very effective method of preventing the creation of TVB-N. A lower average increase of TVB-N was found in carp and trout in MAP with 60% CO<sub>2</sub>+40%

N<sub>2</sub>, than in MAP with a lower percentage of CO<sub>2</sub> (40% CO<sub>2</sub>+60% N<sub>2</sub>) or in vacuum [27]. Examination of several different gaseous mixture (10% O<sub>2</sub>+50% CO<sub>2</sub>+40% N<sub>2</sub>, 10% O<sub>2</sub>+50% CO<sub>2</sub>+40% Ar, 20% O<sub>2</sub>+50% CO<sub>2</sub>+30% N<sub>2</sub>, 20% O<sub>2</sub>+50% CO<sub>2</sub>+30% Ar, 30% O<sub>2</sub>+50% CO<sub>2</sub>+20% N<sub>2</sub> and 30% O<sub>2</sub>+50% CO<sub>2</sub>+20% Ar) on shelf life of trout fillets stored at 1 °C [1] revealed that MAP very effectively prevents the formation of TVB-N, no matter the type of gaseous mixture used. These authors [1] recommended 25 mg N/100 g in trout meat as the highest acceptable limit for TVB-N.

The inhibitory effect of CO<sub>2</sub> on the TVB-N in carp cuts was examined by Milijasević *et al.* [16], who identified the lowest TVB-N occurred in carp cuts in MAP with 100% CO<sub>2</sub>. As concluded by [15], TVB-N in carp steaks was strongly affected by gas atmosphere. During 17 days storage at 3±0.5°C, an increase of TVB-N was observed in all carp steaks, but TVB-N in carp steaks packaged under 90% CO<sub>2</sub>+10% N<sub>2</sub> changed to lesser extent than in carp steaks packaged under 80% O<sub>2</sub>+20% CO<sub>2</sub> and in carp steaks held on top of flaked ice. The basic role of CO<sub>2</sub>, as a gas used for MAP of fish, is to inhibit the growth of microorganisms, in particular bacteria causing the spoilage of food, and even more specifically, those that, with their metabolic activity, give rise to the formation of volatile nitrogenous compounds.

Some researchers have recommended a TVB-N limit from 25 to 35 mg N/100 g as an indicator for rejecting commercial fresh whole fish and processed fish products [28]. However, the European Commission has not established any TVB-N limit for acceptability of common carp. Ježek & Buhtova [29] recommend 20 mg N/100g in carp meat as the highest limit for TVB-N, while [19] recommend the highest limit for TVB-N in carp meat packaged in MAP should be 25 mg N/100 g. Lalitha *et al.* [30] point out that TVB-N value cannot be considered as suitable indicator of fish muscle freshness, because when fish is assessed as sensorially unacceptable, TVB-N does not necessarily exceed the recommended value of 25 mg N/100 g.

## 5.2. pH

A significant physicochemical parameter that affects fish quality is the pH. Numerous authors state the pH of fish is impacted by different factors. However, the development of lactic acid bacteria is the main cause of pH reduction in packaged fish. According to the literature data, a reduction of pH occurs in situations when fish and fish products are packaged in CO<sub>2</sub>-containing gases. On the one hand, this is a consequence of the solubility of CO<sub>2</sub> in tissues, forming carbonic acid that in turn reduces the pH. On the other hand, pH reductions are caused by the antimicrobial activity of CO<sub>2</sub>, which inhibits the growth of microorganisms with metabolic activity leading to the accumulation of base components [31].

pH decreases during storage at 3°C for 21 days were recorded in cleaned trout packaged in MAP with 40%, 60% or 90% CO<sub>2</sub> or under vacuum; at the end of the study period, pH was the lowest in trout with the highest number of lactic acid bacteria, i.e., in vacuum-packed trout [23]. Nevertheless, Milijašević *et al.* [32] proved moderate increases of pH in carp cuts packaged in MAP with 80% O<sub>2</sub>+20% CO<sub>2</sub> after five days of storage; this was explained by the high quantity of basic compounds produced by the activity of fish spoilage bacteria.

## 6. Sensory parameters of importance for shelf life of fish packaged in MAP

Consumers rate fish based on a number of parameters, the most important of which are safety, nutritional characteristics, flavour, odour, colour, texture, convenience for culinary processing and preservation.

Changes in fish meat begin at the moment fish dies, or even earlier, at the time of the catch, and are the result of the activities of the fish's own enzymes, the metabolism of microorganisms and the oxidation of lipids. Changes in the sensory characteristics of fish usually result from proliferation of microorganisms. The decomposition of fish and the growth of microorganisms cause an unpleasant odour and flavour as well as the production of visible pigmented or unpigmented colonies. The synthesis of polysaccharide extracellular materials and diffuse pigments results in sensory changes in the form of mucous formation and discolouration [26]. On the other hand, chemical changes such as auto-oxidation or enzymatic hydrolysis of fats can cause the rise of unpleasant odour and flavour or, in the latter case, the activity of tissue enzymes can lead to unacceptable softening of the fish meat.

The results of numerous studies show that fish packaged in various gas mixtures is, overall, more acceptable from the sensory point of view and, hence, has a longer shelf life than does fish in air or vacuum packed fish. Babić *et al.* [13] showed the highest average rates of overall sensory acceptability, which also proved to be statistically significantly higher, were established for carp cuts packed in an atmosphere consisting of 60% CO<sub>2</sub>+40% N<sub>2</sub>. Carp cuts with somewhat smaller average ratings of overall acceptability were packed in a gas mixture of 40% CO<sub>2</sub>+60% N<sub>2</sub>; the lowest average ratings of overall acceptability were those of vacuum packed carp cuts. Statistically significantly higher sensory ratings during storage were established by Masniyom *et al.* [33] for sea bass fillets packaged in various mixtures of gases than for sea bass held in air, and similar results were obtained by Goulas and Kontominas [7] who examined MAP and vacuum packed mackerel. Such results can also be a confirmation of Murcia *et al.* [34], that food packed in a modified atmosphere retains a more natural and better appearance than does vacuum packed food.

## 7. Perspective of modified atmosphere packaging of fish

The growing need for fresh fish is stoking the development of new technologies to extend fish's shelf life. The use of active packaging and hurdle technology provide more potential for improving the safety and shelf life of fish packaged under MAP. The use of active packaging has recently become increasingly present in the food industry. In active food packaging, packaging materials and the environment interact, including different types of gas emitters and gas absorbers in the process, which results in an extended shelf life [35]. Of the greatest importance for the majority of food types are O<sub>2</sub> absorbers and CO<sub>2</sub> emitters, used either for obtaining a modified atmosphere in the package, or for maintaining a constant atmospheric composition throughout the storage period. O<sub>2</sub> absorbers can be used to maintain a low concentration of O<sub>2</sub> in the package even when materials that are not completely O<sub>2</sub>-impervious are used for packaging [31].

Hurdle technology involves a combination of preservation techniques to create conditions in the food that inhibit the growth of microorganisms. These hurdles can be the storage temperature, water activity, pH, the redox potential, but also newer technologies such as MAP, bioconservation, use of bacteriocins, high-pressure treatment, and the use of edible coatings. Potassium sorbate is used as a preservative to extend the shelf life of fish in combination with modified atmosphere [36].

The use of smart packaging such as time and temperature indicators (TTIs) is a technology with great potential, especially when it comes to products packaged in MAP that require storage in a strictly defined temperature regime. In order to achieve the microbiological adequacy of the product, very rigorous control of the temperature regime is required. The TTI monitors the temperature during storage along the food chain and detects packages that are not kept under the strictly defined cold chain conditions for a certain period of time [37].

## 8. Conclusion

The shelf life of fresh fish can be significantly extended using MAP, but only when the fish is produced with proper control of its hygienic condition and stored at appropriate temperatures. Also, the appropriate selection and use of preservative methods is a very important prerequisite. Only the highest quality fish should be selected to benefit from the extended shelf life advantages of MAP.

Overall, we believe that MAP, if used properly for the right commercial reasons, offers sufficient benefits to both the fishing industry and to consumers, and we strongly advise this is one of many alternatives that the fish industry should consider using as part of a high quality fish marketing program.

Because research data on MAP fresh fish and fishery products has raised safety concerns, additional studies are needed. It is anticipated that future research will help shed light on many unanswered questions and help determine ways to optimise the shelf life extension of fish and fish products, while still maintaining their safety.

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# Antimicrobial growth promoters in feed – possibilities and necessity

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**Abstract.** The attention of the scientific and professional communities, as well as of food consumers, has always been directed towards finding adequate nutritional strategies that could improve food production and safety. By using antibiotics as feed additives, farmers gained increased profits based on higher growth rates with better feed conversion and lower costs of therapeutic treatment. The quantities of antibiotics used as growth promoters for farm animals have been increasing constantly, but at the same time, the frequency of bacterial resistance to antibiotics and the presence of antibiotic residues in food have become a global problem. To eliminate or minimize these risks, on 1 January 2006, antibiotics were banned from use as additives in animal nutrition in the European Union. Accordingly, there is interest in developing new nutritional strategies that would support the function of the autochthonous microbiota in the animal gastrointestinal tract to control pathogenic bacteria. Probiotics, prebiotics, phytobiotics and feed enzymes are a new generation of growth promoters and largely achieve their effects by using the physiological mechanisms of animals and/or their intestinal microbiomes, enabling animals to completely fulfil their genetic potential with respect to production properties.

## 1. Introduction

Since the discovery and application of penicillins in the 1940s, antibiotics have had a major role in the prevention, control and treatment of infectious diseases of humans and animals. It has been proven that the use of antibiotics in the animal feed industry is an important means to increase feed efficiency, promote animal growth, and improve the quality of food of animal origin. The rapid diffusion of antibiotics in almost all areas of feed production and processing was initially considered to be progress and modern technology brought about by modern times. Recent studies have shown that the effect of growth-promoting antibiotics correlated with decreased activity of bile salt hydrolase, an intestinal bacterial enzyme [1]. This decreased enzyme activity has a negative effect on the digestion and utilization of feed by the host animals [1]. By adding antibiotics in small quantities, a positive effect is achieved primarily in animals during growth, although there are data on similar effects in different forms of production. Also, the price of foodstuffs of animal origin is about 8% lower compared to the same product obtained from animals fed with antibiotic-free feeds [2].

With continued use of antibiotics as growth promoters, very quickly (by the late 60s) interest grew, not only in their positive, but also in their possible negative, harmful effects. As a negative effect, the development of resistant strains of enterobacteria is a serious problem for disease treatment in veterinary and human medicine. The problem of resistant strains was also multiplied by the emergence of cross resistance as a result of the adaptive ability of microorganisms and the mutagenic effects of antibiotics. However, unreasonable use of antibiotics has led to the fear of the development of resistant bacteria that could transfer, together with its resistance factors, from animals to humans [3]. Specifically, non-therapeutic antimicrobial use is associated with the propagation of multidrug resistance (MDR),



including resistance to drugs that have never been used on the farm [4]. The next, common, and certainly more significant problem, is the presence of antibiotic residues in food of animal origin, as well as the possible genotoxic action of antibiotics and their residues that are not immediately apparent, but lead to human genome damage in various degrees of character and intensity.

## 2. Legislation

Sweden is the leading country in the prohibition of antibiotics used as growth promoters in animal nutrition in Europe, and in 1986, antibiotics in feed were banned in this country. This new approach to animal breeding did not have a greater reverberation and impact on other European countries until the early 1990s, when it began to strengthen due to the consumer movement and consumerism as a way of thinking and marketing [5]. In 1999, the Scientific Steering Committee conducted screening on the use of antimicrobial substances in animal feed in the EU, and limited the use of antibiotics in animal nutrition. The main goal was to minimize the risk of developing resistant bacteria, and to preserve the effectiveness of the antibiotics that are/were being used in human medicine. In Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives used in animal nutrition, antibiotics, other than coccidiostats and histomonostats, were allowed to be sold and used as feed additives until 31 December 2005 only, and from 1 January 2006, these substances were deleted from the Register.

However, this ban on the use of antibiotics in feed has caused unwanted effects on the animal industry in the EU, such as an increase in infections in animals and a reduction in animal production. In the meantime, the total amount of antibiotics used in animals has increased, as the use of therapeutic antibiotics and disinfectants has increased significantly due to the high incidence of illness caused by the ban. In order to overcome the increased mortality and morbidity rate due to the ban on antibiotics in food, a number of substitutes have been proposed [6]. Alternatives to antibiotics include antibacterial vaccines, immunomodulatory agents, bacteriophages and their lysis, antimicrobial peptides (AMPs), pro-, pre-, sin- and phytobiotics, and feed enzymes [7]. This paper considers the possibility of developing and applying probiotics, prebiotics, phytobiotics and enzymes that are applicable, effective and affordable in mass livestock production as alternatives to antibiotics.

Prevention of disease is defined as the application of a medicinal product to healthy animals in a situation where the specific and increased risk of disease is present [8]. There are key similarities between growth promotion and disease prevention. Many antibiotic alternatives produce both types of positive effects, growth promotion and disease prevention. In many cases, it is likely that the growth-promoting effect is at least partly due to the ability of the product to inhibit or kill bacteria. At the same time, preventing the animals from diseases can prevent loss of productivity due to illness, whether clinical or sub-clinical [9].

**Table 1.** Efficiency of non-antibiotic growth promoters, depending on the animal species and the reason for its use [10]

	Cattle					
	Milk-fed calves	Dairy Cows	Beef cattle	Swine	Chicken	Turkeys
Probiotics	GP, DP	GP, DP	GP, DP, dt	GP, dp, dt	GP, DP	GP, DP
Prebiotics	Gp, dp			gp, dp	GP, DP	GP, DP
Organic acids		gp, dp	gp, dp	GP, dp	GP, DP	dp
In-feed enzymes				GP	GP, DP	GP
Antimicrobial peptides	gp, dp	gp, dp, dt	gp	gp, dp	gp, dp	
Phytochemicals (e.g., essential oils)	dp, dp, dt	Gp	gp	gp, dp	GP, dp	gp, dp

Copper, zinc, and other heavy metals		gp, dp	GP, dp	GP, dp	GP	gp
Immune modulators	DP	DP	DP	gp, dp, dt	gp, DP	gp, dp
Vaccines	DP	DP	DP	GP, DP	DP	DP
Bacteriophages, endolysins, lysozyme, and other hydrolases	Dp	dp, dt		gp, dp, dt	dp, dt	gp, dp

GP - Growth promotion, strong scientific evidence for efficacy and commercially used; DP - Disease prevention, strong scientific evidence for efficacy and commercially used; DT - Disease treatment, strong scientific evidence for efficacy and commercially used; gp - Growth promotion, some scientific evidence suggests potential efficacy; dp - Disease prevention, some scientific evidence suggests potential efficacy; dt - Disease treatment, some scientific evidence suggests potential efficacy

### 3. Feed enzymes

The most encouraging option for promoting growth in animals is the use of enzymes that can be added to feed. These enzymes help animals to digest and absorb plant materials such as cellulose or pectin, which cannot otherwise be used efficiently [11]. Such enzymes (e.g. xylanases and  $\beta$ -glucanases) are usually added to commercial feed for broilers. The mechanism by which the enzymes fulfil the role of growth promoters are not fully understood, but could include changing the intestinal microbiota, preventing damage caused by non-diseased parts of the plants that cause mechanical friction on the mucous membrane, decomposing larger molecules into compounds with prebiotic activity or influencing the composition of the intestinal content and its digestibility [12].

Enzymes in animal feed also have a role in the prevention of some diseases, such as necrotic enteritis in chickens [13]. The most common enzymes used as additives are mixtures of various glycanases. Recombinant synthetic enzymes such as phytase are commercially produced and sold as feed additives for monogastric animals [14]. Phytase has significant effects on the digestibility of calcium, phosphorus and minerals, as well as on the production of intestinal mucin and endogenous losses, all of which affect the supply of nutrients and ejaculation. The dietary use of encapsulated lysozyme, as a supplement in a pullet feed mixture significantly reduced numbers of *Clostridium perfringens* and gastrointestinal lesions in the ileum [15]. The European Food Safety Authority (EFSA) studied a combination of xylanase and  $\beta$ -glucanase and concluded that the product is safe and effective as a growth promoter in broilers and turkeys [16]. Based on numerous studies, including systematic reviews and meta-analyses, it has been found that feed enzymes such as xylanases are effective in reducing intestinal lesions, which are a key pre-disposing factor for necrotic enteritis [17]. In pigs, a high level of acidity in the intestines can inactivate enzymes in feed. Enzymes that are stable under such conditions have shown promising results, pointing to the potential for this alternative strategy as growth promoters. Some enzymes, such as phytase, are more effective in improving performance than others [18]. Feed enzymes are not promising growth promoters for ruminants because the conditions in the rumen inactivate all enzymes before reaching the intestine [19]. It is well known that animal reactions to feed enzymes are not completely predictable, and these inconsistencies can be attributed to the type of enzyme, the amount of enzyme used, the enzyme activity, the composition of the mixture, and the individual characteristics [20].

### 4. Probiotics

Probiotics are live cultures of microorganisms (yeasts, fungi and bacteria) that are used as dietary supplements to improve the balance of the microbiota in the gastrointestinal tract. Probiotics can be divided into defined and undefined. Defined probiotics consist of single strains or mixtures of all-round microorganisms (for example, each microorganism is described at the level of the species, the exact composition of the culture is quantitatively described, and the genomes of particular organisms in the mixture can be completely sequenced to ensure the absence of any antibiotic resistance genes). Undefined probiotics contain microorganisms that are not fully described, usually in mixtures [21].

Generally, undefined probiotics exhibit greater efficacy than defined probiotics, but both strategies are promising approaches to disease prevention, productivity improvement and growth promotion [22].

Numerous scientific studies have quantified the effects of probiotics in growth promotion and disease prevention in broilers. For example, one study showed that probiotics improved bird's productivity and health, and mortality was reduced by more than 20% compared to the control flock [23]. The reduction in mortality was similar to the use of antibiotics [23]. The use of probiotics in laying hens led to a statistically significant increase in productivity [24]. In a study to compare feed enzymes and probiotic strains as additives, both products significantly reduced the mortality of broilers and improved production results compared to animals fed mixtures without additives. Probiotics, however, have shown significantly better results than enzymes in feed. One study has shown there is a wide range of probiotics that can effectively control the clinical symptoms associated with coccidiosis, a potentially destructive disease that is difficult to control without antibiotics [25]. Reviews by the FAO, European Medicines Agency (EMA) and EFSA concluded that probiotics are effective growth promoters in pig breeding and can effectively prevent diarrhoea and reduce mortality due to *Escherichia coli* infections in piglets. Probiotics have also shown efficacy in preventing diarrhoea among young piglets, with a proven reduction in incidence rate of up to 40% [26]. Probiotics have shown efficacy in the prevention of disease in cattle. The use of probiotics increased the efficiency of milk production (measured as kg of milk produced/kg of consumed feed) in dairy cows by 6% [27].

Storage and use of probiotics is a potential challenge. For example, in the production of pelleted feed, the ingredients are usually exposed to high temperatures, which can inactivate probiotics. Although this problem does not exist in other forms of animal feed, probiotics have some common risks, for example potential unwanted and harmful changes in the microbiological balance of the bowel.

## 5. Prebiotics

Prebiotics are non-viable food ingredients that benefit the host by selectively stimulating growth and/or activity of one or a limited number of bacterial species in the digestive tract, thereby improving the host's health [28]. In addition to local, they can exhibit positive systemic effects after resorption of fermentation products of bacterial metabolism. Prebiotics move directly into the colon, where they undergo selective fermentation, and help maintain eubiosis primarily by supporting the preferred microbiota and by increasing excretion of the unwanted microbiota by faeces. Prebiotics help useful microorganisms to proliferate, but could also have other effects, such as immune system modulation. The efficiency of prebiotics seems to be determined by various factors, including the type of prebiotics, age of animal, health status, zoohygienic conditions and farm management, which must be taken into account when deciding whether to use such a strategy.

Among the numerous feed ingredients, non-digestible carbohydrates (oligo and polysaccharides), certain peptides and proteins, and some lipids are potential prebiotics. Due to their chemical structures, these listed feed components are not subject to enzymatic hydrolysis, nor are they absorbed in the frontal part of the digestive tract, so they can be called "colonal foods", that is, food components that move into the last parts of the digestive tract and serve as a substrate for the bacteria present, providing the host with energy, metabolic substrates and essential microorganisms (17). Lactic acid bacteria and bifidobacteria, considered to be the preferred microbiota in the digestive tract, can use carbohydrates originating from prebiotics for the purposes of their metabolism. However, pathogenic bacteria (*E. coli*, *Salmonella* spp.), and many other gram-negative bacteria do not possess these abilities. These bacteria are eliminated from the intestinal microbial population since the preferred bacterial microbiome has more intensive reproduction capacity [29].

Recent reviews by EMA and EFSA concluded that prebiotics are effective in promoting growth and improving the health status of broilers. In pigs, prebiotics have a positive effect on growth, which increases by 8% in the period after piglets are rejected. In pigs fed with prebiotic supplements, immune responses to intestinal infections such as salmonellosis have been improved with prebiotics [30]. In cattle, prebiotic efficacy appears to be limited to calves. The addition of prebiotics to milk substitutes (galactosyl-lactose) for calves was efficacious [31].

## 6. Phytobiotics

Phytobiotics are a relatively novel group of additives that have attracted considerable attention in recent years in the feed industry. Phytogetic animal feed additives (phytobiotics or plant drugs) are defined as compounds of plant origin used in animal nutrition to improve animal production results, feed quality, and the quality of the resultant food of animal origin. The said definition is based on the manner of use, while other terms such as herbs, spices, essential oils (EOs) and oily resins can be used in the classification of a wide range of phytogetic compounds. Compared with synthetic antibiotics and inorganic chemicals, these products are obtained from natural sources, are less processed, do not create residues, and could become ideal animal additives and successfully replace antibiotic growth promoters in feed [32]. The basic groups of secondary metabolites in plants include alkaloids, heterosides, saponins, tannins and terpenoids. In the terpenoids, mono- and sesquiterpenes are produced (these form the basis of EOs), while aromatic phenylpropane ingredients are also present in smaller quantities in plants.

Paracelsus von Hohenheim first used the term “essential oil” in the fourteenth century, and the term referred to an effective component of a drug, a “quinta essentia” [33]. Over 3000 types of EOs are known, of which 300 are commercially important and used in industry as aromatic substances. EOs are volatile, scented substances with an oily consistency, produced by plants. The main components of EOs, aldehydes or phenols (cinnamaldehyde, citral, carvacrol, thymol, eugenol), exhibit the highest antibacterial activity, while EOs containing terpenic alcohols have somewhat lower activity. Since EOs are composed of a large number of ingredients, it is presumed that their antimicrobial activity is not related to a specific mechanism of action but is directed at several different targets in the microbial cell. The operating modes of EOs are cell wall degradation, cytoplasmic membrane damage, membrane protein damage, cell cell loss, cytoplasm coagulation, and depletion of the proton gradient [34]. EOs are believed to produce antibacterial effects through two different mechanisms: the first one is related to their hydrophobicity which allows the EO to be imprinted on the phospholipid bilayer cell membrane, while the other relates to the inhibition of bacterial enzymes and receptors by EO binding at specific sites. Thanks to their hydrophobic structure, EOs are then able to destabilize and change the permeability of the bacterial membrane.

## 7. Conclusion

Various products may be able to replace antibiotics used for the purposes of growth promotion and disease prevention, but implementing these products will require a comprehensive approach ready to consider these alternatives as a part of farm management. All in all, alternatives to antibiotics are promising, as many of them seem to increase animal productivity and simultaneously prevent infection. The attention of the expert and scientific communities is directed towards the replacement of antibiotics in many animal welfare situations. This should allow antibiotics to be preserved for use only when necessary for health protection purposes in humans or animals. Based on numerous examples from on-farm practice and scientific studies, the use of growth promoters other than antibiotics in animal nutrition is medically, nutritionally and economically justified.

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# Risk assessment and risk management of contaminants in the feed to food chain

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**Abstract.** In feed production processes, factories usually produce different mixtures within the same production line. Consequently, remainders of the first-produced feed can stay in the system and be mixed with the following feed charge. This type of transfer (carry-over) is unavoidable in the production systems currently used, and thus, non-medicated feed can be contaminated with veterinary drugs present in a previously manufactured charge of medicated feed. The carry-over of veterinary medicinal products is associated with the risk of residues remaining in the tissues of treated animals at the time of slaughter and poses a health hazard to consumers. Producing safe feed and food products is, first and foremost, a question of good management practices at each stage of the feed and food chain, from primary production to final processing. Primary responsibility for feed safety rests with the feed business operator, who must ensure that all stages of production, processing and distribution under their control are carried out in accordance with relevant legislation, good manufacturing practice and principles contained in the HACCP system. Concrete steps for feed manufacturers to prevent drug carry-over are using one or more approved cleanout procedures of manufacturing equipment, such as cleaning, flushing or sequencing.

## 1. Introduction

The ‘farm-to-fork’ approach promoted by the European Union requires the assessment and control of major components of the food production chain, with emphasis on primary production. Feeds must satisfy the nutritional requirements of the relevant animal species, and they are expected to support safe and cost-effective production of foods of animal origin, as well as to ensure the welfare of farm animals [1]. Adequate animal feedingstuffs, which are the main input into livestock production, should be used to ensure the final product reaching the market has the required quality and poses no risk to the consumer [2].

The competitiveness of the agricultural sector because of globalization has led to the need for intensified productivity of animal production systems. For this reason, the stocking rate in poultry and pig production units was increased, causing a greater frequency of disease due to higher infection pressure [3]. Although in recent years much emphasis has been placed on disease prevention through improved management and environmental conditions, intensive animal production systems still depend on drugs (antimicrobials), as shown by their continuously growing market [4]. A common means to deliver such drugs is by including them in feed, as the large-scale use of feedlots makes this easy, and as it avoids handling animals for individual drug administration. However, Dunlop et al. [5] and Varga



et al. [6] found the use of antimicrobials in feed results in a higher incidence of bacteria resistant to the active ingredients, when compared to parenteral treatments.

Also, the benefit of improved productivity from the use of veterinary medicinal products (VMPs) in food producing animals is accompanied by the risk of VMP residues remaining in the tissues of treated animals at the time of slaughter or residues in animal-derived products; such residues pose a health hazard to consumers. The major public health significances of drug residues are development of antimicrobial drug resistance, hypersensitivity reaction, carcinogenicity, mutagenicity, teratogenicity, and disruption of normal intestinal microbiota [7]. Currently, microbial antibiotic resistance is considered to be one of the greatest threats to human health. In the United States, more than 2 million people are infected with antibiotic-resistant bacteria annually, with 23,000 deaths being the direct result [8].

VMPs are critically needed to meet the challenges of providing adequate amounts of food for the growing world population [9], but they should not be used habitually to prevent disease or to compensate for poor hygiene or inadequate husbandry conditions. All antibiotics, including those administered in feed, must be prescribed by the veterinarian responsible for the animals concerned. In order to conserve the effectiveness of antibiotics in the future, it is important that wherever and whenever these medicines are used, they are used responsibly: "Use as little as possible and use as much as necessary" [10]. Even when feed containing veterinary drugs is prepared following good manufacturing practice guidelines, carry-over of active ingredients from previous formulations cannot be ruled out, because most of the mixed feed formulations are prepared in multiproduct plants. Carry-over contamination can occur during the whole production process [11], with obvious serious consequences. Risk assessment and risk management of contaminants in feeds is ultimately a key issue for veterinary public health.

## **2. Legal aspects of veterinary drug carry-over in feeds**

After production of medicated feed, it is very difficult to completely avoid carry-over of drugs into feed that should be free from such substances (zero tolerance). From the standpoint of legislation, the veterinary drug residues in feed are not allowed and their presence, determined during official monitoring, excludes the placing of such feed on the market. Currently, this principle applies to eight countries within the EU.

However, there are alternative ways to avoid zero tolerance: direct use of orally-administered drug in powder form on the farm, top dressing or administration of drugs *via* drinking water. Each of these methods has certain application risks. Oral powders are usually not dosed into feed by specific, calibrated, devices, but are dosed manually by farmers, with evident weaknesses in such a process. The use of top dressing methods risk that strong, dominant, animals achieve over-intake, while weaker animals with less access to feed achieve lower intake than expected. In such a scenario, the target microbial pathogen in an animal is exposed to subtherapeutic dosage of the antimicrobial, so some significant number of the target pathogens survives treatment. This induces selection of drug-resistant microbial pathogens. The imprecision of delivering drugs *via* the drinking water system is reflected in the amount of water spilled and the variation in the amount of water the animals actually drink. Practical drawbacks are the creation of solid complexes in the pipes and obstruction of drinking nipples, which affect the precision of drug dosing [12].

Most countries in the European Union do not have clearly established national limits for unwanted carry-over of veterinary drugs in non-target feed, while three countries have established limit values, primarily based more on the ALARA values (As Low As Reasonably Achievable), rather than risk assessment for public health:

1. In Belgium, ALARA values are applied, but only under the conditions that the level of cross-contamination cannot cause: a) animal health disorder; b) exceeded MRL in products of animal origin, or; c) increased antimicrobial resistance. In particular, the upper limit values for contamination should never be above 2.5% of the minimum prescribed dose for antibiotics or 3% of the maximum prescribed dose for antihelmintics.

2. In France, validation of the production process is applied: the maximum permitted contamination by VMPs is 5% in the first and 1% in the second charge after production of the last medicated feed batch.

3. The legal status in the Netherlands is still in the process of adoption, but the maximum permitted contamination in non-target feed will be up to 2.5% of the lowest dose of VMP permitted in the targeted feed.

Stolker et al. [13] have shown that the percentage of veterinary medicine carry-over (in the production of medicated feed for pigs in the Netherlands) is not correlated with the percentage determined by standard Good Manufacturing Practice (GMP+) procedures. More precisely, it is not possible to predict the concentration of antibiotic in a flushing charge based on determined percentage of carry-over in the feed production plant. The inability to avoid carry-over, non-homogeneity in the production of medicated feed, and the previously stated difficulties in predicting the level of carry-over, along with increasing concern about the growing problem of microbial resistance, motivated NEVEDI, an association of Dutch feed manufacturers, to announce that they would voluntarily stop the production of medicated feed in 2011, which was the first case of this kind in Europe.

In Serbia, the Rulebook on the Quality of Feed [14] states (in Article 88) that feed mixtures must not contain antibiotics or sulphonamides, i.e. zero tolerance is applied, so these substances must not be present in feedingstuffs.

### **3. Legal aspects of carry-over of coccidiostatics and histomonostatics**

In addition to carry-over of veterinary medicines, special attention is required to understand regulatory aspects concerning the presence of coccidiostatics and histomonostatics in non-target feed. Coccidiostats and histomonostats are substances intended to inhibit the growth or destruction of protozoa, and these substances can, *inter alia*, be approved for use as feed additives in accordance with European Regulatory Council Regulation (EC) No 1831/2003 [15]. It can be a confusing fact that some coccidiostats are registered not as feed additives but as drugs, i.e. VMPs. For active substances in the VMP that are the same as a substance in a feed additive, the applicable maximum level of cross-contamination in non-target feed is the maximum content of feed additive in complete feed established in the relevant Union act [16]. A list of the named coccidiostats (registered as VMPs) is given in the Annex of Allowed Substances in Commission Regulation No. 37/2010 [17] and consists of Amprolium, Decoquinat, Diclazuril, Halofuginone, Imidocarb, Lasalocid and Toltrazuril. As such, they can be used in the production of medicated feed, based on veterinary prescription. They are most commonly used in the breakthrough of coccidiosis, where no coccidiostats are added in feed, in cases of development of resistance, or when the vaccines are insufficiently efficacious. Carry-over of coccidiostats and histomonostats can lead to contamination of feed where the use of coccidiostats or histomonostats is not authorized, such as feed for animal species or categories not specified in the authorization of the additive. This inevitable cross-contamination can occur at every stage of production and processing of feed, as well as during storage and transport of feed. Inevitable transfer of active substances contained in approved coccidiostats and histomonostats into non-target feeds results in the presence of undesirable substances in the feed in accordance with Directive 2002/32/EC [18]. Thus, taking into account the application of good manufacturing practice, the maximum level of unavoidable carry-over should be established according to the ALARA principle. In order to allow the feed producer to manage the inevitable transfer, a transfer rate of about 3% (in relation to the maximum allowed content) should be taken into account in terms of feed for less sensitive animal species, while for feed intended for sensitive non-target species and feed with a withdrawal period, i.e. feed used in the pre-slaughter period, a transfer rate of about 1% can be considered. A transfer rate of 1% should also be established for the cross-contamination of other feed for the target species when it has no added coccidiostats and histomonostats, as well as for non-target feed for animals such as dairy cows or layer hens, where clear evidence exists of transfer from feed to food of animal origin. In the European Union, this described problem is regulated by Commission Directive 2009/8/EC of 10 February 2009 [19]. In that Directive, the maximum level of imminent carry-over in non-target feed for 11 coccidiostats has

been set: Lasalocid sodium, Narasin, Salinomycin sodium, Monensin sodium, Semduramicin sodium, Maduramicin ammonium alpha, Robenidine hydrochloride, Decoquinate, Halofuginone hydrobromide, Nicarbazine and Diclazuril. The levels are expressed in mg/kg (ppm) in feed with a moisture content of 12% (Table 1) and are aimed at avoiding excessive exposure of animals to these compounds, since most of the compounds have a relatively low safety margin and their higher concentration in feed can cause harmful effects even in the target animal species. In Serbia, in accordance with the harmonization of the regulations regarding the animal feed safety sector, in the Rulebook on the Quality of Feed [14], Article 99 (maximum permissible harmful substances) established identical levels as the EU has for these above-mentioned 11 coccidiostats, while the manner of their use and their maximum residue levels in food are given in Article 89. When interpreting this Rulebook, it is necessary to note that most coccidiostats have been registered as additives only until 2018, so the time limit for placing them on the market has already expired. However, other coccidiostats have been registered as additives for longer (until 2020, 2021, 2022, 2023, or 2025), and so all these compounds must be carefully selected for use in feeds on the basis of their being registered as additives or not.

**Table 1.** Maximum content levels for coccidiostats in feed as laid down in Commission Directive 2009/8/EC and Council Regulation 2010/37/EC (Adopted from O'Mahony et al., [20]).

Feed additive	Feed dose (mg/kg)	Non-target feed (mg/kg)		Withdrawal feed (mg/kg)
		Sensitive	Other species	
Lasalocid sodium	125	1.25	3.75	1.25
		D, C, R, E, DA, LB, T > 12 wks, CL > 16 wks		CF, CL < 16 wks, T < 12 wks
Narasin	70	0.7	2.1	0.7
		T, R, E, LB, CL > 16 wks		CF
Salinomycin sodium	70	0.7	2.1	0.7
		E, T, LB, CL > 12 wks		CF, CL < 12 wks, RF
Monensin sodium	125	1.25	3.75	1.25
		E, D, SR, Du, B, DC, LB, CL, T > 16 wks		CF, CL, T < 16 wks
Semduramicin sodium	25	0.25	0.75	0.25
		LB, CL > 16 wks		CF
Maduramicin NH <sub>4</sub> alpha	5	0.05	0.15	0.05
		E, R, T, LB, CL > 16 wks		CF, T < 16 wks
Robenidine HCl	70	0.7	2.1	0.7

		LB, CL > 16 wks		CF, RFB, T
Decoquinat	40	0.4	1.2	0.4
		LB, CL > 16 wks		CF
Halofuginone HBr	3	0.03	0.09	0.03
		LB, CL > 16 wks, T > 12 wks	Except CL < 16 wks	CF, T < 12 wks
Nicarbazin	50	0.5	1.5	0.5
		E, LB, CL > 16 wks		CF
Diclazuril	1	0.01	0.03	0.01
		LB, CL > 16 wks, TF > 12 wks	Except CL < 16 wks, CF, TF < 12 wks	RFB

Key: D = dogs, C = calves, R = rabbits, E = equine, DA = dairy animals, LB = laying birds, T = turkeys, TF = turkeys for fattening, CL = chickens reared for laying, CF = chickens for fattening, RF = rabbits for fattening, RFB = rabbits for fattening and breeding, SR = small ruminants (sheep and goats), Du = ducks, B = bovine, DC = dairy cattle.

#### 4. Risk assessment of carry-over

The primary responsibility for feed safety rests with the feed business operator, who must ensure that all stages of production, processing and distribution under their control are carried out in accordance with relevant legislation and good manufacturing practice [21]. The feed producer (commercial or farm) is obligated to ensure that the exact amount of the desired drug is correctly incorporated and that there is no cross-contamination of any unwanted drug in that feed.

In feed production processes, factories produce different mixtures within the same production line. Consequently, remainders of the first-produced feed can stay in the system and be mixed with the following feed charge [19]. This type of transfer is unavoidable in the production systems currently used, and thus, non-medicated feed can be contaminated with veterinary drugs present in a previously manufactured charge of medicated feed [2]. Carry-over is (usually) expressed as the percentage of the nutrient, veterinary medicament and/or contaminant from one feed batch that ends up in the following feed batch (flushing charge). Stolker et al. [13] have documented that sometimes a flushing feed is contaminated not only with the antibiotic used directly before the production of the flushing feed, but also with an antibiotic used several batches earlier in the production process. The same authors pointed to the importance of testing the homogeneity of the flushing charge, i.e. determining whether the drug is homogeneously distributed in the feed. They found [13] that during the first 20 minutes in the production cycle, the flushing charge contained oxytetracycline at concentrations >2.5% of the allowed transfer, or significantly higher than the last part of the produced batch. If the flushing charge can be easily flushed, the concentration of the contaminant from the previous batch will be very high at the beginning and will rapidly fall, but in contrast, in the case of slow flushing, the concentration of the given substance will only gradually decrease [22]. These data must be taken into account when taking the first kilogram of this type of feed and giving it to a non-target (for the antibiotic-sensitive) animal species, as well as possible errors in the interpretation of results if a sample of such feed is sent for further analysis.

The type of drug (category I or II), the number of animal species for which the drug is intended, and the feed delivery system determine the degree of risk associated with carry-over. Feed plants producing

feed only for one type of animal and using only Category I drugs (which do not have a withdrawal period) have the least risk of contamination and the occurrence of residues in tissues of non-target animals. Since there is no withdrawal period for these products, they can be used until animals are slaughtered, and subsequent animal products can immediately be released to the market. In contrast, carry-over of Category II drugs (which have a withdrawal period) into feed could result in the unwanted presence of residues in meat, dairy products and eggs of animals. The carry-over of drugs classified as either Category I or Category II into a batch of feed intended for a species the drug is not intended for can create serious health problems for any such animal consuming the feed [23]. The carry-over of Monensin from cattle feed to horse feed which can result in lethal outcomes. The high sensitivity of horses to Monensin is associated with their lack of demethylation enzymes, which facilitate the clearance of Monensin from the animals' systems [24]. The U.S. Food and Drug Administration issued warning letters in 2018 for two feed mills in Minnesota, and Nebraska that mixed horse feed containing monensin. These firms did not adhere to Current Good Manufacturing Practice (CGMP) requirements for medicated feed mills. These incidents of monensin toxicity should be a reminder to all feed producers that make medicated feeds that they must remain vigilant in adhering to CGMP requirements by eliminating unsafe carry-over of medications into feed intended for different species. Guidance for Industry # 72 (GMPs for Medicated Feed Manufacturers Not Required to Register and Being Licensed with FDA) and Guidance for Industry # 235 (Current Good Manufacturing Practice Requirements for Food for Animals) are documents that provide explanation and examples of how to meet the FDA's requirements for safe animal feed production.

Carry-over can appear in one segment of the production line or can be a result of a combination of residues along the whole system [2]. In order to discover the cause of carry-over, all equipment must be taken into account, from the place of delivery of the medicine to the loading zone, but carry-over occurring during transport or on the farm itself must also be considered. O'Keeffe et al. [25] identified multiple potential causes for the presence of coccidiostat (Nicarbazine) residues in edible livestock tissues: contamination of feed in mixtures and/or during transport, supply of wrong feed, delivery of feed to the wrong bin on the farm, inadequate cleaning of the feeding system on farms before delivery of replacement feed, inadequately applied withdrawal period for feed with coccidiostatics, poor farm management that led to re-exposure of poultry to Nicarbazine in the period immediately prior to slaughter, and fecal recycling of Nicarbazine from the litter. McEvoy et al. [26] also pointed to the importance of particles of dust and excess material remaining during the pelleting process as an important factor in carry-over. The production practice at one factory was such that material returned to pre-press and contaminated the next production lot [26]. The most important sources of carry-over, related to production equipment, are summarized in Table 2. In Good Manufacturing Practice guidance [27], the main causes of carry-over are the dosing/grinding/mixing line, the press line, and the measurement stations within the lines. This type of carry-over is termed installation carry-over.

**Table 2.** Sources of carry-over regarding feed plant equipment (Adopted from Harner et al., [23])

Equipment	Mode of Carry-over
Dust system	-delayed return of dust to production line
	-excessive pickup of drug and carrier
	-hang-up (electrostatic or moisture)
Mixer	-residual feed remaining in mixer

	-buildup of material on ribbons and walls
	-electrostatic hang-up on walls and top
	-leaking mixer gate (not fully closed)
	-incomplete clean-out
Surge bin	-electrostatic or moisture hang-up
Conveyors	-same as surge bin
	-residual feed remaining in buckets and boot
Elevators	-electrostatic or moisture hang-up
	-bridging
Bins	-residual feed from incomplete cleanout
	-error in bin chart records
Bulk truck	-incomplete clean-out
	-bridging and hang-up

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## 5. Risk management of carry-over

When the sources of carry-over are revealed, corrective measures can be taken. The basic principles that feed business operators should establish, implement and maintain [21] are contained in the HACCP system. HACCP principles are largely limited to the ability to carry out the following:

- (a) Identify any hazards that must be prevented, eliminated or reduced to acceptable levels;
- (b) Identify the critical control points at the step or steps at which the control is essential to prevent or eliminate a hazard or reduce it to acceptable levels;
- (c) Establish critical limits at critical control points which separate the acceptability from unacceptability, for the prevention, elimination or reduction of identified hazards;
- (d) Establish and implement effective monitoring procedures at critical control points;
- (e) Establish corrective action when monitoring indicates that a critical control point is not under control;
- (f) Establish procedures to verify that the measures outlined in points (a) to (e) are complete and effective. Verification procedures will be carried out regularly;
- (g) Establish documents and records commensurate with the nature and size of the feed business to demonstrate the effective application of the measures set out in points (a) to (f).

HACCP principles can help feed business operators to achieve a higher standard of feed safety, but should not be considered as a method of self-regulation and do not replace official controls. Each plant must establish its own rules and manage carry-over based on their own HACCP program. Although most feed businesses are familiar with ISO 9000, it focuses on systems and procedures. However, HACCP is different, as it focuses on the product. ISO 9000 and similar standards are not an essential requirement for successful HACCP programs [28].

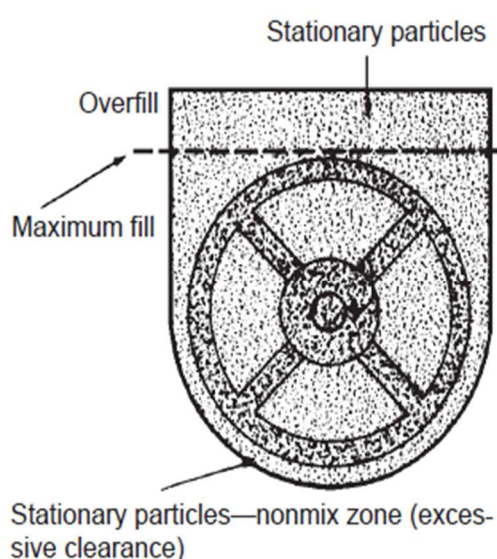
Concrete steps for feed manufacturers to prevent drug carry-over are set by CGMP requirements, and they involve using one or more approved cleanout procedures for the manufacturing equipment,

such as cleaning, sequencing and/or flushing [29]. The FDA's CGMP requirements serve as guidelines for medicated feed manufacturers to ensure that their products meet identity, strength, and quality standards.

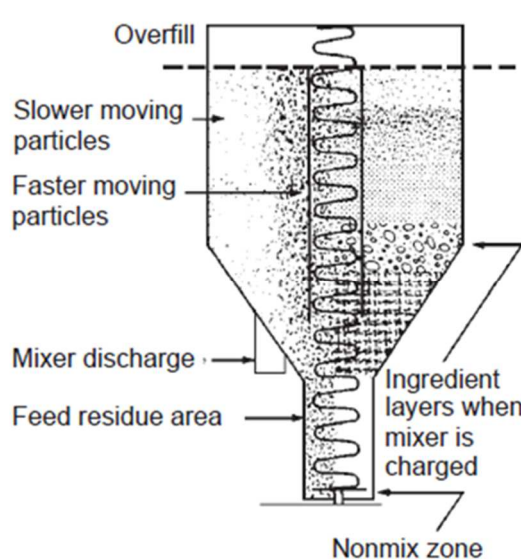
### 5.1. Cleaning the equipment

Equipment cleaning is still not widespread in the feed manufacturing industry, but it is potentially the most effective method of avoiding carry-over during processing and delivery of feed. It is mainly applied in high risk situations: dealing with medical premixes; when sequencing can not be included in the production schedule; with portable grinder-mixers; when the physical properties of the drug are such (adhesion strength and electrostatic properties) that sequencing and flushing do not prevent carry-over, and; if liquid ingredients (molasses or fat) are added during the feed mixture production [23]. In all of these cases, physically cleaning the production equipment (cleaning of the mixer, transport system, pellet coolers, bins) or delivery trucks is required. This involves completely stopping production, which is impractical and economically burdensome for the factory. However, GMP stipulates that all equipment should be designed, constructed, installed and maintained in such a way as to facilitate the inspection and use of cleaning procedures. In terms of cleaning efficiency, horizontal mixers have an advantage over vertical ones. A typical cross-section of a double-ribbon horizontal mixer is shown in Figure 1. When the mixer has been emptied, the residual feed will remain in the space between the outer ribbon and the housing. Some mixers can be adjusted to reduce this space to about 6 mm, which reduces the feed carry-over to an innocuous level in most cases. A typical single screw vertical mixer is shown in Figure 2, along with the location of the mixer discharge. A considerable amount of feed will remain in the mixer after the last feed leaves the discharge opening. If a clean-out opening is provided down on the boot of the mixer, the residual feed can be removed there. If it is not removed, a significant amount (18 kilos or more) of carry-over can pass into the following feed charge [30]. Some plants clean their equipment routinely, e.g. at monthly or bi-annual levels. When employees perform cleaning tasks, it is important to remove all waste and residues before the next production cycle, use safe and approved cleaning agents, use safe and clean tools, pay attention to the hygiene of the personnel involved in the task, and ensure that washing does not disseminate microbial or other contaminants in the equipment.

After cleaning, inspection should safely review all available parts of production line (such as mixers, containers, conveyors, etc.) to ensure they are clean and that carry-over will not occur. Inspection should be carried out visually, without entering the mixer or bin [31].



**Figure 1.** Double ribbon horizontal mixer from Wilcox et al., [30])



**Figure 2.** Single screw vertical mixer (Adopted from Wilcox et al., [30])



### 5.2. System flushing

The flushing procedure involves passing a precise amount of a selected ingredient through the system to flush through any residual medicated feed produced in the previous batch. As a flushing material, grain meals are often used, most commonly ground corn of approximately 600 microns. Other suitable material that has been proven to adequately clean the production line can be used [23, 32], as can wheat [28], limestone [31], and rice hulls [33]. When material passes through the production system, it is mixed with the residual medicated feed from the previous batch, and dilutes the drug concentration to a safe level. The quantity of flushing material depends on the system; it usually amounts to about 5-10% of the mixer capacity and should not be less than 90 kilograms [23]. Some tests have shown that flushing material amounting to 1-2.5% of the mixer capacity can be effective in preventing carry-over [32]. Also, the plant should check with mixer's manufacturer for their recommendation for flushing material type, and choose the best option. Due to the degree of variability among facilities, feedmills should determine their facility's individual characteristics and apply appropriate time and volume requirements for flushing material to accomplish the intent of the procedures. The volume used should be stated in the written procedures, and should be based on documented analysis or tests of the firm's system [31].

After the flushing material is added to the mixer, the mixer should be allowed to operate for at least 1 minute before the material is removed. After the mixer is flushed, the material should pass through the whole production system along the same pathway the previously manufactured medicated feed passed. The flushing material, from that moment, must be correctly identified and stored in order to prevent contamination, and later it can be used in the production of the same medicated feed. Some plants use this flushing material to flush trucks for bulk deliveries after they make deliveries to the farm. When applying these procedures, the economic implications of the need to store the flushing material should be taken into account. Some companies choose to simply discard this material in order to avoid subsequent possible production errors, which is certainly the economically least attractive option. Manufacturers must document the applicable flushing procedures: the flushing method, the flushing time, the amount and type of flushing material and the disposal of the flushing material.

To monitor flushing, inspection must ensure that feed producers adequately apply their own procedures. It is necessary to check that the entire system is flushed (including mixer, conveyors, bins) and to visually determine whether any foreign material is not in accordance with the flushing material. Finally, quantity of flushing material released into the system must be present at the end of the flushing process [31].

### 5.3. Sequencing procedure

The feed industry most often uses sequencing because it minimizes discontinuation of the production line. If properly planned and executed, this method is the most cost-effective carry-over prevention procedure. The order in which the feed is prepared, processed and delivered directly determines the probability of carrying over the drug from one to the next batch and, consequently, the presence of residues in the tissues of the animals that consume such feed. This work plan involves production of all medicated feeds that contain the same drug in the sequence from the highest to the lowest level of the drug. After completion of the last batch of medicated feed, the production of non-medicated feed for the same animal species continues.

Examples of accepted principles to be considered when designing the sequencing process [34] are:

- Withdrawal feed and feed for cattle should not be produced and processed in the same equipment after the manufacture of medicated feed containing Category II drugs, unless appropriate cleaning procedures are applied. Drugs with specific toxicity characteristics, such as Monensin's toxicity for horses, require special attention.
- After the production of medicated feed containing Category II drugs, feed for the same species that are below the marketable age or weight may be produced.
- Feeds that have high potential for dangerous drug contamination (feed for withdrawal, dairy animals, etc.) should be produced first in the series, and feed with the most toxic drugs will be the last in the sequencing process, followed by complete physical cleaning of the system.

- Sequencing procedures and practices should be clearly understood by all persons responsible for the planning and production of medicated feed. They should be easily accessible for their use.

During the sequencing of feed, the age of the animal, the sensitivity to the administered drug, and the type and purpose of the drug should be considered. For example, after production of medicated feed containing oxytetracycline for broilers, non-medicated feed for layers should not be produced. Given that a very small amount of sulfamethazine consumed by pigs can lead to residues of this drug in meat, it is not acceptable to manufacture feed for pigs after the production of medicated feed with sulfamethazine [29]. Feed for pigs containing Carbadox should not be followed by unmedicated feed for gravid sows. After production of Monensin-containing feed, only non-medicated feed for cattle, poultry, or pigs can follow, but not feed for horses. If feed is produced for only one type of animal, such as pigs, the most common medicated feed is for the youngest, most vulnerable category, in this case piglets. In this case, the following order is applied: first, feed containing a drug that requires a withdrawal period for piglets, then sow, grower and finally, finisher feed. Sequencing can also be used to clean containers on trucks, but the same principles should be followed. In most feed factories, feed sequencing procedures will reduce carry-over to a level that eliminates the potential for the presence of residues in animal tissue. However, the sequencing procedure cannot reduce carry-over to a sufficiently low level unless the problems listed in Table 2 have been previously resolved. When sequencing is applied, in order to avoid cross-contamination, precise records of feed production documentation are imperative, so the last batch in a series can always be safely marked. Otherwise, the sequencing procedure could be compromised by the next feed charge preparation. Periodic evaluation of sequencing procedure should be carried out to verify and validate their effectiveness [27, 28, 31].

After the application of the described cleaning procedures, if the undesired carry-over of critical additives and VMPs can still be expected, then the company could take up the following measures: draw up a mandatory production (working) sequence; additional measures in the event of product changes; produce feeds with critical additives and VMPs on another line; switch to less critical agents [27]. When carry-over is detected in a feed plant, harmful effects can occur in people and in animals that consume contaminated feed. This type of failure is considered a violation of the regulations, with all consequences for the responsible persons. Based on the Rules on the Establishment of the Feed Safety Monitoring Program for 2018 [35], in Serbia, if the presence of contaminants in feed is detected, activities ranging from corrective measures to prohibition of operation and closure of the entire feed production facility will be implemented.

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# Comparative antioxidant study of onion and garlic waste and bulbs

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**Abstract.** Among vegetables, onion and garlic production occupies a leading position worldwide due to their wide usage in various sectors of the food industry. Husk is the main waste from onion and garlic processing. The biological properties of onion and garlic bulbs are well studied, but the husks are not well investigated to date. The total antioxidant capacities of aqueous and ethanol extracts of onion and garlic husks and bulbs were determined by the ORAC method and expressed in  $\mu\text{mol equiv. Trolox/g}$  of sample. The husks demonstrated the greatest total antioxidant capacities and significantly exceeded those of the bulbs. Onion husk was the most active, and its total antioxidant capacity was  $521.24 \pm 11.23 \mu\text{mol equiv. Trolox/g}$  of sample for aqueous extract and  $1206.93 \pm 8.37 \mu\text{mol equiv. Trolox/g}$  of sample for ethanol extract. Antioxidant capacity depended on extractant type.

## 1. Introduction

The biological properties of fruit and vegetable wastes are intensively studied due to the need to reduce environmental pollution [1], as well as to search for new sources of natural antioxidants. To date, it has been determined that the peel of many plants contains large numbers of antioxidants, including polyphenols [1-3]. Therefore, agricultural and industrial wastes are attractive sources of natural antioxidants and dietary fibers [1].

Onions (*Allium cepa*) and garlic (*Allium sativum* L.) are well known vegetables, traditionally used in food processing and medicine [4,5]. Onion and garlic are the most commonly cultivated vegetables, worldwide. According to the Food and Agriculture Organization (FAO), onion is grown in 126 countries on 3.6 million hectares, producing 73.20 million tons per year [4]. Garlic is the second most widely cultivated vegetable after onion. The FAO estimates 1.217 million hectares are used worldwide to cultivate garlic, producing 16.41 million tons per year [5].

Fresh, fried, sautéed, boiled, salted and marinated onion and garlic are used in the food industry in recipes for canned meat, spicy sauces, side dishes, as well as in fish, sausage and pickled cheese processing [4]. Dried onion is also widely produced and used alone or as part of vegetable mixes as flavoring additives in snacks, soups, sauces, gravies, minced meat and fish semi-finished products [6].

In addition to food purposes, onion and garlic are used in other areas, including medicine, due to their biological properties, such as antibacterial, anticancer, hypoglycemic, hypolipidemic, antiplatelet aggregation, and antioxidant activities [4,7,5,8].

Husk is the main waste in *Allioideae* spp. processing. Thus, the amount of waste from peeling onions is from 5.0-9.0% to 21.6-29.9% of feedstock weight depending on the size of the bulbs and the



method of peeling [9], which is approximately 3.66-21.9 million tons per year. The amount of waste from peeling garlic is 16-20% of feedstock weight [9], which is approximately 2.3-2.9 million tons per year.

The aim of this study was to examine and compare the total antioxidant capacity (TAC) of aqueous and ethanol extracts of onion, garlic and their husks, as well as to assess the competitiveness of the most active extracts in comparison with popular spices and herbs from the OracDataBase.

## 2. Materials and methods

Onion (*Allium cepa*) and garlic (*Allium sativum* L.) bulbs and husks were studied. Aqueous and ethanol extracts of bulbs and husks were prepared for the TAC study. The raw materials were ground to less than 5 mm in size and mixed with distilled water at 95°C for aqueous extracts, or with 75% ethanol at room temperature for ethanol extracts, then infused for 15 minutes and 24 hours, respectively, and filtered through a paper filter. Aqueous extracts were prepared directly on the day of the experiment, and alcohol extracts were stored at 4°C for 3 days. Table 1 presents the ratios of extractant and vegetable material used.

**Table 1.** The ratio of extractant vegetable material.

Type of extractant	Husk	Bulb
Distilled water	1:100	1:25
Ethanol	1:25	1:5

The TAC of extracts was determined by the oxygen radical absorbance capacity (ORAC) fluorescent method [10] with the author's modification on a Fluoroscanner Ascent FL system (TermoLabsystems, Finland) using black 96-well plates. Volumes of 30 µL of sample or standard and 200 µL of 0.5 µM of sodium fluorescein (Sigma-Aldrich, USA) were added to the wells, microplates were covered with film (SSIbio, USA) and placed into the Fluoroscanner Ascent FL (TermoLabsystems, Finland) for 30 min at 37°C. Then, 30 µL of 153 µM AAPH (Aldrich Chemistry, USA) was added to each well, and fluorescence was measured at 37°C for 60 min at 5 min intervals. Excitation wavelength was 485 nm, emission wavelength was 535 nm. The TAC of each sample was determined four times.

TAC was determined according to a standard curve using (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (Sigma-Aldrich, Switzerland) in the concentration range of 5-75 µM. Depending on their activity, the extracts were diluted with 75 mM phosphate buffer (pH = 7.4). TAC was expressed in µmol equiv. Trolox/g of sample.

STATISTICA 10.0 software was used for statistical analyses. The results were calculated as mean ± standard error (M ± SE). Significant differences were tested by one-way ANOVA, followed by Tukey's test. Differences with P-values less than 0.05 were considered as statistically significant.

## 3. Results and discussion

Results are presented Table 2. The husks demonstrated higher TACs than did the bulbs. Onion husk displayed the greatest TAC, 521.24 ± 11.23 µmol equiv. Trolox/g of sample for aqueous extract and 1206.93 ± 8.37 µmol equiv. Trolox/g of sample for ethanol extract, and these exceeded the TACs of garlic husk by 503.08-fold (P<0.05) and 1060.29-fold (P<0.05), respectively.

**Table 2.** Total antioxidant capacity (TAC) of vegetables and vegetable waste extracts

Sample	TAC of aqueous extract (mean ± SE; µmol equiv. Trolox/g)	TAC of ethanol extract (mean ± SE; µmol equiv. Trolox/g)
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Onion husk	$521.24 \pm 11.23^a$	$1206.93 \pm 8.37^{b,c}$
Onion bulb	$9.60 \pm 0.78^{b,d,e}$	$41.45 \pm 1.37^{b,d,f,g}$
Garlic husk	$18.16 \pm 0.92^{b,d,h,i}$	$146.64 \pm 4.54^{b,d,f,h,j,k}$
Garlic bulb	$16.64 \pm 0.73^{b,d,h,l,m}$	$45.15 \pm 0.74^{b,d,f,j,l,n}$

a-b, c-d, e-f, g-h, i-j, k-l, m-n – significant differences between the samples ( $P < 0.05$ )

Substances having antioxidant activity are mainly organic compounds; therefore, yields from raw material should be higher in the case of organic solvent extraction due to the chemical affinity. Casagrande *et al* [11] compared the antioxidant activity of plant samples depending on solvent used. The results of their study [11] showed the efficiency of solvents increases in following order: water < ethanol < acetone. Smetanska *et al* [12] studied five different organic solvents: acetone, methanol, ethanol, hexane and diethyl ether. The results of their study [12] showed the antioxidant yield significantly depends on the polarity of organic solvents; methanol and ethanol produced better yields of phenolic compounds [12]. In addition, ethanol is the most suitable solvent for extracting compounds with biological activity that can be included in food, pharmaceutical or cosmetic products, since ethanol possess the lowest toxicity among the organic solvents and produces high yields [11].

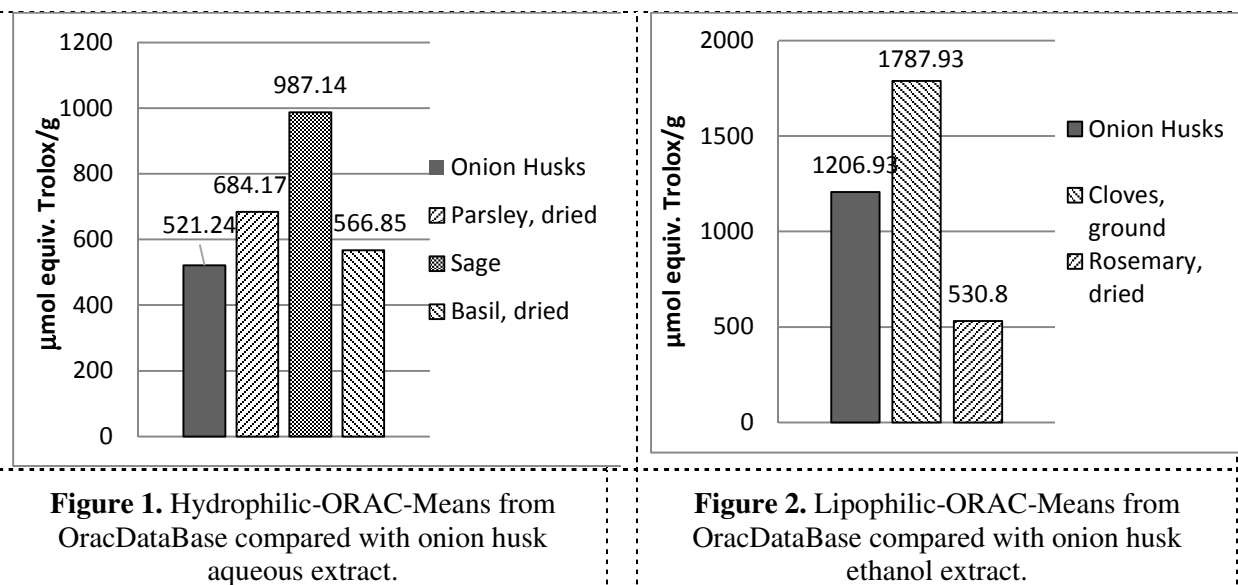
In the current study, both aqueous and alcoholic extracts of the bulbs displayed smaller TACs than did the corresponding husk extracts.

When comparing aqueous extracts of garlic husk and bulb, there were no statistically significant differences ( $P > 0.05$ ), but the TAC of onion husk extract exceeded the TAC of onion bulb by 54.3-fold ( $P < 0.05$ ). The TAC of garlic husk ethanol extract was higher than the TAC of garlic bulb ethanol extract by 3.2-fold ( $P < 0.05$ ), while the TAC of onion bulb extract was 29.1-fold greater than that of onion husk ethanol extract ( $P < 0.05$ ). There was no statistically significant difference ( $P > 0.05$ ) between the TACs of garlic and onion bulb ethanol extracts, while the TAC of garlic bulb aqueous extract exceeded the TAC of onion bulb aqueous extract by 1.7-fold ( $P < 0.05$ ). The obtained TACs of garlic bulb extract were consistent with the data described by Deng *et al* [13]; TACs of our onion and garlic bulbs also coincided with the results of Morales-Soto *et al* [14]. However, there are few works devoted to the study of onion and garlic husk antioxidant capacity.

The obtained TACs of all extracts were compared with data from the international OracDataBase, created in 2007 by the USDA (United States Department of Agriculture) [15]. Hydrophilic-ORAC-Means for onion and garlic bulbs were 16.94 and 55.41  $\mu\text{mol equiv. Trolox/g}$  of sample, respectively, and were slightly different from the TACs obtained in the current study.

As a result of our research, the most promising raw material was onion husk, but this is not listed in the OracDataBase; therefore TAC of onion husk was compared with various herbs and spices rich in antioxidants [16,17] and traditionally used in the food industry as natural preservatives [18,19].

Parsley (dried), sage, basil (dried) possessed the highest Hydrophilic-ORAC-Means, while cloves (ground) and rosemary (dried) had the highest Lipophilic -ORAC-Mean. Comparison of some spices and herbs with onion husk is presented in Figures 1 and 2. The TAC of onion husk aqueous extract is about half the Hydrophilic-ORAC-Mean of sage, but has about the same TAC as parsley and basil. The TAC of onion husk ethanol extract exceeded by 2.8-fold the Lipophilic-ORAC-Mean of the well known source of natural antioxidants, dried rosemary, but at the same time, it was 32.5% less than the Lipophilic-ORAC-Mean of cloves.



The biological properties of onion and garlic bulbs, including their antioxidant potentials, have been intensively studied for a long time [4-6]. However, our study revealed that husks demonstrated higher TACs than bulbs; onion husk was the most promising raw material. Moreover, the affinity of the solvent significantly affected the yield of compounds with antioxidant activity.

#### 4. Conclusions

This study confirmed onion husk to be a good source of antioxidants, as its TAC was  $521.24 \pm 11.23$  μmol equiv. Trolox/g of sample for aqueous extract and  $1206.93 \pm 8.37$  μmol equiv. Trolox/g of sample for ethanol extract, which exceeded the TAC of onion bulb aqueous and ethanol extracts by 54.3- and 62.2-fold, respectively.

The TAC of onion husk was approximately equivalent to the TACs of well-studied herbs and spices such as sage, parsley, basil and rosemary that are used in the food industry. Therefore, onion husk could compete with them and be used as alternative source of antioxidants instead of synthetic antioxidants. It is also worth noting that the onion husk is a waste of the vegetable industry, so its use would be ecologically sound.

However, it is necessary to study the composition of onion husk in order to explain the different TACs of aqueous and ethanol extracts. Rationalization and selection of suitable extraction parameters, including type of extractant, sample-solvent ratio and temperature, among others, is required.

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# Postmortem glycolysis and pork quality

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**Abstract.** After an animal is harvested for meat, the skeletal muscle initiates a myriad of biochemical pathways in an attempt to maintain energy homeostasis. Anaerobic glycolysis is responsible for the generation of ATP to help meet energy demand and for the decrease in pH by generating H<sup>+</sup>. Both the rate and the extent of the *post-mortem* pH decline are paramount in the context of the development of pork quality attributes, such as color, water holding capacity, and texture. Pale, soft and exudative meat and dark, firm, and dry meat are two of the major quality defects facing the pork meat industry. Because glycolysis has the potential to affect meat quality attributes either positively or negatively, evaluating its regulation *post-mortem* is fundamental to understanding meat quality. Therefore, the aim of this study was to evaluate factors that affect mechanism of glycolysis. Special consideration will be given to meat quality attributes and development of pork quality defects.

## 1. Introduction

During the *post-mortem* period, a myriad of energetic, biochemical, and physical changes takes place in the muscle that results in its conversion to meat. As the animal succumbs to exsanguination and resulting anoxia, skeletal muscle still attempts to achieve its *ante-mortem* homeostatic balance through adenosine triphosphate (ATP) synthesis and its utilization. ATP is synthesized through the catabolism of stored glycogen to ultimately produce lactate [1]. The end products of ATP hydrolysis and *post-mortem* glycolysis, hydrogen ions (H<sup>+</sup>) and lactate, respectively, accumulate in the muscle due to the lack of an effective elimination mechanism. This accumulation of H<sup>+</sup> ions acidifies the muscles and consequently causes the pH to drop from 7.2 in living muscle to an ultimate pH (pHu) of approximately 5.5 [2]. Under normal circumstances, muscle pH declines gradually until the onset of *rigor mortis*. Any alterations in the rate or extent of pH decline in *post-mortem* muscle can have substantial influence on meat quality. Hastened, extended, or insufficient *post-mortem* pH decline adversely influences meat color, water-holding capacity (WHC) and texture and can lead to occurrence of two classical fresh meat quality problems in the pork meat industry: pale, soft and exudative (PSE) meat and dark, firm and dry (DFD) meat [1,3]. PSE pork is characterized by pale color, low WHC, and soft texture. Conversely, DFD meat has a dark color and a firm, dry, and sticky texture due to its enhanced water binding capability [1]. A greater understanding of *post-mortem* metabolism during the conversion of muscle to meat and the mechanisms that govern glycolysis are critical to characterizing pork quality development. Therefore, the aim of this study was to summarize current understanding and new discoveries in the *post-mortem* regulation of glycolysis and their impacts on meat quality.

## 2. The conversion of muscle to meat

The biochemistry behind the transformation of muscle to meat is a heavily researched topic. The basic biochemical reactions and physical changes underlying the conversion of muscle to meat are well recognized. As ATP is utilized and depleted, actomyosin cross-bridges become permanent and the muscle extensibility decreases. This loss of extensibility signifies the onset of *rigor mortis*. The



magnitude, extent, and timing of *post-mortem* metabolism can dramatically affect meat quality development. The rate of pH decline during the conversion of muscle to meat reflects the intensity of *post-mortem* metabolism. Many factors influence the rate and the extent of the *post-mortem* pH decline, including intrinsic factors such as species, the type of muscle, anatomical location, carcass fatness, and variability between animals, and extrinsic factors such as animal feeding, pre-slaughter handling, the environmental temperature. Typically, pH gradually declines from around 7.2 to around 5.7 within 8 h *post-mortem*, with pHu of about 5.6 achieved at 24 h [1, 4].

### 3. *Post-mortem* glycolysis in pigs

One of the key metabolic pathways in the conversion of muscle to meat is glycolysis. Under anaerobic conditions, glycogen is catabolized to glucose 6-phosphate through glycogenolysis. Glucose 6-phosphate is the initial substrate in the glycolytic pathway (glycolysis), a sequence of ten reactions, all of which occur in the sarcoplasm of the muscle fiber [5]. Glycogen is the primary and possibly sole carbohydrate source utilized in the rephosphorylation of adenosine diphosphate (ADP) in *post-mortem* muscle [2]. One glycogen moiety generates three ATP molecules and two lactate molecules *post-mortem*, which is why glycogen has been studied for its role in determination of pHu and meat quality [2]. Classically, glycolysis is considered to generate H<sup>+</sup> and lactate at a 1:1 ratio, but this does not necessarily mean the H<sup>+</sup> is derived from the dissociation of lactic acid [6, 7]. Within the past decade, new theories have ignited a debate challenging the notion of 'lactic acidosis' [7]. Once pyruvate is formed by glycolysis, it is converted to lactate under anaerobic conditions. The conversion of pyruvate to lactate by lactate dehydrogenase serves as a buffer by removing a H<sup>+</sup> ion from the system and attaching it to the lactate molecule, thus limiting muscle acidification [7, 8]. Rather, the hydrolysis of ATP produces the H<sup>+</sup> ions responsible for *post-mortem* acidification during the conversion of muscle to meat [5]. Lactate was also investigated to predict pHu and meat quality [9, 10, 11, 12]. High concentrations of exsanguination lactate were associated with lower meat quality such as decreased WHC, lighter color [9], and lower pH 45 mins *post-mortem* [10].

#### 3.1. Regulation of rate and extend of glycolysis

While most authors believe glycogen depletion arrests pH decline, some suggest that glycogen content alone cannot fully explain the limitation in pH decline [3]. This raises the question, why does glycolysis stop? Although the most logical reason is depletion of the muscle's glycogen depot, metabolizable carbohydrate is rarely depleted entirely in muscles [13]. The extent of *post-mortem* metabolism is dictated by the glycogen content of the muscle as long as levels are between 0 and 53 mmol/kg muscle, and for normal *post-mortem* pH decline, the minimum content of glycogen is about 53 mmol/kg muscle [14]. Further increase in glycogen levels beyond 53 mmol/kg muscle is not associated with further decline in pH [14]. Muscles with limited glycogen reserves, which were reduced below normal levels at slaughter due to *ante-mortem* stress, usually have limited pH decline and resultant elevated pHu (>6). The resulting meat can exhibit inferior quality attributes, like a dark color and reduced overall acceptability. Thus, when reduced, muscle glycogen content can affect the extent of *post-mortem* pH decline and meat quality. The role of glycogen content in dictating normal pHu is less established when glycogen is not reduced [2]. When oxidative muscles were provided with excess glycogen in an *in vitro* glycolytic system, they still produced a high pHu compared to glycolytic muscles [3]. Therefore, according to some authors, glycogen is not an exclusive explanation for higher pHu (near 5.8-6.0), but rather, muscle glycolytic capacity, i.e. the maximal ability of a cell to convert carbohydrate (i.e. glycogen) to pyruvate or lactate, controls the extent of pH decline in pig muscles [3, 15]. Hence, evaluation of enzymes regulating glycolytic capacity is critical to further understand the meat quality attributes developed during *post-mortem* transformation of muscle to meat [2]. According to some authors, phosphofructokinase-1 (PFK-1) is the most likely candidate for regulation of *post-mortem* glycolysis [2, 13]. Glycogen can be converted to lactate and H<sup>+</sup>, provided PFK-1 maintains its activity. This enzyme begins to lose activity around pH 5.9 and becomes completely inactive at pH 5.5, which could explain why the pHu of pork is bracketed in a fairly

consistent range between pH 5.5-5.9 [2, 13]. However, PFK-1 inactivation does not explain why some muscles produce meat with a high pHu (pH > 5.9) in the presence of residual glycogen [2]. Further investigation of factors regulating PFK-1 activity *post-mortem*, such as adenonucleotide availability (ATP and AMP) and AMP deaminase abundance and activity, could clarify the regulation of glycolysis and *post-mortem* pH decline [2, 3, 16]. Recent evidence increasingly suggests that mitochondrial content and function both likely influence *post-mortem* metabolism and variability of pHu within a species [17, 18].

#### 4. Meat quality attributes

Meat quality has always been important to consumers and is a particularly important issue for the meat industry of the 21st century. The innate characteristics of muscles and extrinsic conditions during slaughter influence the development of three key traits important to fresh meat quality: WHC, color, and texture. Fresh meat quality is a broad area of investigation encompassing many topics that include glycolysis and *post-mortem* pH. pHu is widely regarded as an indicator of fresh meat quality [19]. The normal pHu value of pork ranges between 5.5 and 5.7 [4]. Meat with pHu < 5.4 has a pale color, lower WHC, reduced protein extractability, and poor processing yield, while meat with pHu ≥ 6.0 appears darker in color and has a shorter shelf life.

##### 4.1. Color

Color is considered the most important sensory attribute of fresh meat, and is what consumers primarily use as an indicator of quality and freshness of the products [20]. The distribution and amount of myoglobin species, deoxymyoglobin, oxymyoglobin and metmyoglobin, together with internal reflectance influence the color of the pork [21]. Fresh meat color is also significantly influenced by *post-mortem* metabolism. Abnormally low pHu causes denaturation of muscle proteins, including myoglobin, and reduces their ability to bind water. As a result, large amounts of water migrate from inside the muscle fibers to the extracellular space, which increases light reflectance and results in a paler meat color [22]. On the contrary, high pHu causes meat color to appear darker due to greater WHC and increased light absorbance, lower reflectance, and lower protein denaturation. High pHu also promotes greater activity of oxygen-scavenging enzymes, resulting in reduced available oxygen to bind myoglobin, and more deoxymyoglobin formation [23].

##### 4.2. Water-holding capacity

The ability of pork to retain its inherent moisture has a dramatic impact on consumer acceptance of fresh pork. WHC is an important quality attribute as it influences the yield and the quality of fresh and processed meat products [24]. Many factors, such as pH and *post-mortem* proteolysis, influence WHC by altering the amount and location of moisture in muscle [24]. Rapid pH decline coupled with high muscle temperature in early *post-mortem* causes the denaturation of approximately 20% of muscle proteins, leading to their loss of functionality and their ability to hold water [24]. WHC is closely related to the color of meat and influences other physical properties including texture and firmness of raw meat and eating properties of cooked meat [25].

##### 4.3. Texture

Texture is a complex concept that involves several attributes including, tenderness, juiciness, firmness, and cohesiveness. *Post-mortem* factors that contribute to the texture of the meat include *post-mortem* pH, carcass temperature, contractile state, proteolysis, and their interactions [5]. Meat tenderness decreases as pHu increases from 5.4 to 6.0, and then improves as pHu increases from 6.0 to 7.0 [26, 27]. Meat texture is also affected by the rate of pH decline. Rapid pH decline increases protein denaturation and decreases WHC, resulting in an undesirable soft texture in pork.

#### 5. Abnormal *post-mortem* metabolism and meat quality defects

The meat industry has focused selection pressure on producing animals that are efficient feed converters, fast growing and have high lean meat content. These characteristics have been achieved through genetic manipulation and careful selection of breeds [22]. However, this focus has also resulted in the production of animals that are much more susceptible to stress, and consequently, to the development of meat quality defects, ranging from PSE to DFD pork [12]. Due to the considerable economic losses, PSE and DFD pork have attracted significant research attention.

#### *5.1. Pale, soft, and exudative meat*

PSE meat is characterized by an abnormally light color, soft texture, and impaired ability to hold water. Hastened glycolysis coupled with a corresponding increase in  $H^+$  accumulation and heat production causes rapid pH decline when carcass temperature is still high [22]. The resulting meat is pale in color due to the loss of meat pigments or the oxidation of heme pigments, and due to the increase in light reflectance at meat surface [22]. This meat is exudative as a result of impaired WHC, and is soft in texture because of protein denaturation [22]. The development of PSE is mainly associated with genetic factors and pre-slaughter stressors including improper handling, and mixing with unfamiliar animals [12]. Acute stress immediately prior to slaughter can also accelerate glycogenolysis and glycolysis resulting in PSE meat [28]. Under stressful situations, epinephrine is secreted from the adrenal medulla and functions primarily to raise blood glucose levels through the stimulation of glycogen degradation [29].

#### *5.2. Dark, firm and dry meat*

DFD meat is characterized by its dark color, firm texture, and dry sticky surface. When animals are exposed to chronic or long-term stress before slaughter, DFD meat can result. Chronic stress prior to slaughter leads to the depletion of stored glycogen, so less glycogen is available *post-mortem*, affecting the normal process of acidification and leaving the meat pH high [22]. The high pHu (> 6.0) results in relatively little denaturation of proteins, water is tightly bound, and little or no exudate is formed [30]. High pHu minimizes meat pigment losses and denaturation, thereby increasing light absorbance, which gives the meat its darker appearance [22]. Meat with elevated pHu also exhibits higher WHC as the pH is further from the isoelectric point than normal [22].

### **6. Conclusions**

The rate and extent of *post-mortem* glycolysis has a profound effect on pork quality. Therefore, understanding mechanisms controlling *post-mortem* glycolysis and pH decline should improve the probability of producing high-quality pork meat. Recent research in this area further elucidated the biochemical mechanisms regulating glycolysis and improved the understanding of *post-mortem* muscle glycolysis and meat quality development. Despite this, further efforts are necessary to explain the rate and extent of *post-mortem* pH decline in order to ensure consistent production of the highest quality meat possible. Also, those involved in the meat industry must understand these biological processes and implement management practices that optimize them.

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## Meat quality: Impact of various pre-slaughter conditions

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**Abstract.** Poor animal welfare is a growing issue of concern all over the world. During pre-slaughter phases, some activities, if poorly performed, lead to stress reactions in animals. Animals' responses to a great extent depend on species, breed, sex and age of animal. Food and water deprivation, exposure to unfavourable environmental conditions, improper handling and social mixing are just some indicators of compromised animal well-being. All these detrimental activities lead to the development of carcass and meat quality defects. Among them, important defects are untimely death of animals, body weight loss, carcass damage, and the two major meat quality defects, i.e. pale, soft, exudative (PSE) and dark, firm, dry (DFD) meats. Consumer dissatisfaction results, and with economic and financial losses, producers are placed in a disadvantageous position.

### 1. Introduction

Over the past few decades, meat consumption has significantly increased throughout the world [1]. Customer demands for meat safety and quality are high, but at the same time, there are great opportunities and demands for lower meat prices [2]. Meat quality can be defined from the aspect of its functional or conformational attributes. Functional characteristics are desirable and include yield and gross composition, appearance and palatability, while conformance attributes are linked precisely with customers' perceptions [3]. In this respect, important visual traits are texture and colour of meat, and colour, amount and distribution of fat. When the meat is cooked, customer satisfaction is largely determined by meat tenderness, flavour, odour and juiciness [4].

In general terms, each meat trait is under single or multiple gene control. However, expression of genotype depends to a great extent on environmental conditions, which can differ. These conditions, from the aspect of meat quality, include various pre-slaughter conditions and post-slaughter factors. Interactions between genes and environment occur to a greater or lesser degree, and it is difficult to separate each of them [5]. The pre-slaughter phase involves all activities and processes of animals from a farm to the slaughterhouse. These activities are carried out on farm, market, during transportation and at the slaughterhouse. If any of these phases performs poorly, animal welfare will be compromised. Furthermore,





stress reactions then occur, resulting in negative changes to carcass and meat quality [6]. Thus, the aim of this paper is to point out some pre-slaughter conditions that can reduce animal well-being and meat quality.

## 2. Pre-slaughter stressors

During their lifetime, animals are exposed to various stressful situations. How they respond largely depends on species, breed, sex and age of animal. Ruminants are more resistant to stress than poultry and pigs. Females and young animals are more susceptible to stress than males and older animals [7]. The animal's previous experience with handling on-farm could influence further behaviour [6, 8]. For instance, pigs will be easier to move if they have been correctly handled earlier [9].

After their growth period, animals are sent to the market, or directly from farms to the slaughterhouse. Transportation can have negative impacts on animal health, well-being and performance as well as on food safety and carcass and meat quality. Stress situations cause injury, mortality and morbidity, and are consequences of food and water deprivation during the transportation period, exposure to noise, vibrations and toxins, exposure to variable climate conditions, poor handling and mixing with unfamiliar groups [10]. For cattle, handling procedures during loading and unloading have been described as major stressors [11]. The effects of stress on animal can vary according to various factors such as the quality of the procedure, handlers' experience, the quality of the handling equipment and animal health conditions [12]. Experience of the transport drivers also plays an important role. Body weight loss of animals at unloading was lower in cattle transported by drivers who had 6 or more years experience than in those driven by people with 5 years or less experience [13].

At the markets, animals are usually kept in open pens and can be in mixed groups. Exposure to these negative environmental conditions, starvation, water withdrawal and social dominance are just some of the factors that can affect the physiological integrity of an individual animal. At the slaughterhouses, animals rest in lairage before slaughter in order to fill glycogen reserves that have been harvested during earlier pre-slaughter activities. Despite this positive effect, lairage can be a main source of meat quality problems. Animals can gain bruises and injuries as a result of fighting among themselves, being beaten poorly trained workers or overcrowding [7]. The lairage acts as a reservoir of pathogenic microorganisms, and excessive retention of animals increases the risk of carcass contamination [14].

## 3. Affected carcass and meat quality traits

All of these stressful *ante-mortem* operations lead to the development of carcass and meat quality defects. Among them are mortality, carcass damage, shrink loss, contamination by pathogens, and pale, soft, exudative (PSE) and dark, firm, dry (DFD) meat. Untimely animal death is considered as the worst situation that could happen from the financial aspect, due to the total loss of the carcass. Carcass damage such as bruising, haemorrhages, skin blemishes and bloodsplash are clear signs of improper handling procedures. All these damages cause extravascular accumulation of blood and, therefore, can serve as a potential medium for microbial growth, allowing accelerated meat spoilage. Trimming off the affected parts decreases meat yield and value and increases costs and processing time [3].

PSE and DFD meats are two major problems faced by the meat industry. Previously, PSE was linked with pigs and DFD with all meat animal species. However, it can be considered that both conditions occur in all animal species in correlation with how the animals were treated before slaughter [15]. Stress is manifested from all pre-slaughter processes and depending on its form, one of these meat defects can develop. PSE is caused by acute stress. Ordinarily, detrimental activities just before the start of slaughter such as the use of electrical goads, hitting animals, or the animals fighting among themselves cause this phenomenon in meat [3]. The accelerated rate of *post-mortem* glycolysis is followed by the rapid decline of pH while the temperature of the meat is still high [16]. Meat with pH lower than 6.0 measured 45 minutes after slaughter or with ultimate pH <5.3 is labelled as PSE [3, 17, 18, 19]. On the other hand, chronic stress

conditions induced by too long transportation, starvation and high stocking density at lairage during a long time lead to the occurrence of DFD meats [3]. Due to a small amount of glycogen *post-mortem*, the normal process of acidification is slowed down, keeping the pH of meat high. Meat with ultimate pH >6.0 can be considered as DFD [19, 20, 21, 22]. Both of these conditions are undesirable to consumers because of the poor meat quality and low processing quality for further processed products [23].

#### 4. Conclusion

Activities and processes before slaughter are essential factors associated with carcass and meat quality. Improper pre-slaughter conditions affect animal welfare, leading to the deaths of animals, body weight loss, carcass damage, and PSE and DFD meats. In addition, along with consumer dissatisfaction, producers are placed in a disadvantageous position due to their economic and financial losses.

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# The feasibility of pulsed light processing in the meat industry

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**Abstract.** Today, the increasing demand for minimally processed foods that are nutritious, sensorially acceptable, and free from microbial, chemical and physical hazards, challenges research and development to establish alternative methods to reduce the level of bacterial contamination. As one of the newly developing non-thermal methods, pulsed light is a technology for the fast, mild, and residue-free surface decontamination of meat and meat contact materials in the meat processing environment. This review provides specific information on pulsed light technology and the feasibility of its application for unpackaged and packaged meat and meat products as well as meat contact materials. The advantages, limitations and achieved effects of pulsed light on microbial inactivation, lipid peroxidation, sensory quality and color of meat, seafood and meat products are illustrated and discussed in relation to its implementation on the industrial level.

## 1. Introduction

Food safety has become an essential priority for authorities and consumers worldwide, especially concerning perishable products such as those of animal origin. Considering meat as an excellent substrate for microbial growth, numerous thermal and non-thermal decontamination and preservation methods have been developed in order to sustain its safety and quality. Here, next to other food processing technologies—e.g. high pressure, pulsed electric field [1], osmotic dehydration, radio frequency electric field, ultrasound, irradiation, and chemical and biochemical hurdles (e.g. organic acids, enzymes, plant derived antimicrobials)—pulsed light (PL) application promises to reduce the likelihood of food being a vector for bacterial infection and toxin production [2]. In addition, different sources describe PL, also known as intense light pulses (ILP), pulsed white light and pulsed UV light, as a fast and mild alternative decontamination method that retains the natural appearance of the foods while being of energy saving and of environmental interest. It is based on the application of short time light pulses with an intense broad spectrum [3]. PL includes the employment of inert gas flash lamps to transform short duration as well as high power electric pulses into short duration and high-power pulses of radiation having similar spectrum to that of the sun (200–1100 nm), including infrared (IR), visible light (VL) and ultraviolet (UV). To adequately characterize the effects of PL, basic measures should be taken into consideration, including, but not limited to fluence (F) [ $\text{J m}^{-2}$ ] that describes the total radiant energy that is received from the light source by the matrix per unit area during the treatment time (in seconds) and the number of pulses (n) applied during the whole treatment [2].



These pulses inactivate the microorganisms at the surface level of food and the packaging material [4]. UV light is absorbed by the microbial DNA, which leads physico-chemical changes in its structure, damaging the genetic information and resulting in impaired replication and gene transcription as well as eventual cell death [5]. The objective of the present review is to provide current and practical information on the feasibility of PL treatments for meat and meat products in terms of bacterial inactivation. However, since non-thermal methods of food preservation are being developed to eliminate or at least minimize the quality degradation of foods that result from thermal processing, special attention will be paid to the effect of PL on sensory quality and color of different varieties of meat, meat products including game, poultry and seafood.

## 2. Microbial inactivation

In general, around 2.0 log CFU/mL reduction of the initial microbial count was achieved after PL treatments, independently of the target microorganism and the matrix. *Listeria monocytogenes* and *Salmonella enterica* were the main studied microorganisms in fish after applying PL treatments. Overall, similar values of *L. monocytogenes* decontamination were found in both meat (0.9-2.24 log CFU/mL) and fish/seafood (0.7-2.4 log CFU/mL), whereas for *S. enterica* the values were ~ 2.0 log CFU/mL [6]. Moreover, Hierro *et al.* [7] assessed the feasibility of PL treatments (0.7, 2.1, 4.2, 8.4 and 11.9 J/cm<sup>2</sup>) to enhance the safety of beef and tuna carpaccio. The results indicated a significant reduction in the initial microbial count (~1 log CFU/cm<sup>2</sup>) of the samples inoculated with *Vibrio parahaemolyticus*, *E. coli*, *L. monocytogenes* and *Salmonella* Typhimurium after applying PL treatments (8.4 and 11.9 J/cm<sup>2</sup>), and obtained a significant improvement in the food safety of these products.

The results obtained in the investigation of Rajkovic *et al.* [8] demonstrated that under all tested conditions, PL treatment was equally effective in inactivation of all pathogens (*E. coli* O157, *Staphylococcus aureus*, *L. monocytogenes*, and *Salmonella* spp.) inoculated on the surface of the fermented salami slices, with a maximum microbial inactivation of ~2.2 log CFU/g. The overall difference in the mean log reduction achieved, between all pathogens, was smaller than 0.2 log CFU/g, which is in agreement with the findings of Gomez-Lopez *et al.* [9] who did not observe any difference in the susceptibility among different groups of microorganisms, after studying 27 different bacterial, yeast, and mold species. In contrast, research of Ganan *et al.* [10] reported that *S. Typhimurium* showed a slightly higher resistance to PL than *L. monocytogenes*, although these differences tended to disappear at the highest fluences assayed.

The results of Rajkovic *et al.* [11] also demonstrated that under all tested conditions, PL treatment was equally effective ( $P > 0.05$ ) in inactivation of both *L. monocytogenes* and *E. coli* O157:H7 inoculated on the surface of the slicing knife. The overall difference in the mean log reduction achieved, between the two pathogens, was less than 0.1 log (N0/N), i.e. mean log reductions were 4.57 log (N0/N) for *L. monocytogenes* and 4.62 log (N0/N) for *E. coli* O157:H7.

## 3. Lipid peroxidation

In general, continuous UV light induces intensive lipid peroxidation due to the longer exposition time necessary to achieve a suitable light dose for effective inactivation of surface microorganisms in meat. By its nature, PL is a very intensive UV light, from which the lipid peroxidation in treated meat can occur. Lipid peroxidation is a very important factor because it usually causes meat deterioration [12]. However, in the investigation of Rajkovic *et al.* [8], both vacuum and modified atmosphere packed samples of sliced fermented salami were PL treated before they were packed and kept under refrigerated storage conditions for 9 weeks. The fermented salami slices, untreated (control) and PL treated, were analyzed for lipid oxidation changes after the first day and 3, 6 and 9 weeks of cold storage. Immediately after the PL treatment, on the first day of cold storage, dry fermented salami kept in vacuum showed no significant difference in the concentration of malondialdehyde (MDA) between the control (0.33mg MDA/kg), 3 J/cm<sup>2</sup> (0.31mg MDA/kg) and 15 J/cm<sup>2</sup> treated samples (0.49mg MDA/kg). Similar results were noted for the salami kept in modified atmosphere. The MDA concentration in salami kept both in vacuum and modified atmosphere, in all three salami groups (control, 3 J/cm<sup>2</sup> treated and 15 J/cm<sup>2</sup>

treated), continually increased during the cold storage, but a significant difference ( $p < 0.05$ ), compared to the values obtained after the first day of storage, was observed only after 9 weeks of storage. The highest MDA concentration (1.23mg MDA/kg) in all of the salami samples investigated for lipid oxidation was observed in ready-to-eat dry fermented salami (modified atmosphere, treated with 15 J/cm<sup>2</sup>) after a period of 9 weeks [8]. Similar results were reported for chicken meat [13], chicken frankfurters [14] and vacuum packed ham slices [15] by other researchers. Koch *et al.* [16] also did not find any association of PL treatment with lipid peroxidation, due to low oxidation level induced by PL treatments on pork skin ( $<0.12 \mu\text{g/g}$ ). When applied in pulsed form, the short duration of the pulse limits oxidative changes in lipids due to the short half-life of the p-bonds, which prevents efficient coupling with oxygen [17], and which could explain the low 2-thiobarbituric acid-reactive substance levels observed in PL treated meat and meat products.

#### 4. Sensory quality

In the research of Tomasevic [18], PL treatment did not significantly change appearance and total score values of beef. The color score also remained unchanged regardless of the level of fluence applied, which was in contrast of the findings of Hierro *et al.* [19], where the color of beef was assessed by panel members as slightly lighter after the treatment of 11.9 J/cm<sup>2</sup>. The application of 1-pulse (3.4 J/cm<sup>2</sup>) in [18] significantly decreased the score for beef odor, while this happened only after 8.4 J/cm<sup>2</sup> was applied to beef carpaccio [19]. The beef odor was, however, still assessed as acceptable in both studies even after the highest fluency rate was applied. According to the results of Tomasevic [18], the odor of beef meat is a little more sensitive to PL than the odor of pork. For poultry, the only sensory attribute affected by PL treatment was odor but not to an extent that could affect the pondered average values of the total sensory quality for the chicken and turkey meat [18]. A similar finding was published by Paskeviciute *et al.* [13], where UV light dose higher than 6 J/cm<sup>2</sup> had only a moderate effect on the odor of chicken. The odor scores significantly decreased in all game meat samples after 17 J/cm<sup>2</sup> treatment; this was most easily observable in deer meat, and essentially contributed to the significant change of its pondered average value of total sensory quality. The effect of the treatment on odor was the least pronounced in kangaroo meat [18].

Tomasevic [20] also reported that the 17 J/cm<sup>2</sup> treatment resulted in significant quality degradation in two ready-to-eat cooked meat products evaluated. The sensory quality of Parisian sausage and cooked ham deteriorated after the 17 J/cm<sup>2</sup> treatment to such an extent that they were assessed as unacceptable products, with unpleasant odor similar to the one found in scalding facilities in slaughterhouses, terrible taste (regardless of smell), atypical yellowish and brownish color, strong aftertaste and poor texture [20]. These findings are quite the opposite of what was previously reported by Hierro *et al.* [21], where the test panelists did not find significant differences in any of the parameters evaluated among PL and non-PL ham slices. According to [20], dry-cured meat products, Parma ham and bacon, showed greater resistance to the effects of PL than the cooked meat products examined. There were no statistically significant differences in any of the attributes evaluated between 1-pulse treated and untreated samples of Parma ham [20]. In the case of bacon, the same treatment caused significant difference only in odor, although assessors noted that the odor of both, treated and control bacon, was not so pronounced. When the higher fluence of 17 J/cm<sup>2</sup> was applied to Parma ham and bacon, their odor and taste significantly decreased to the level of neither good nor poor, as assessed by the panelists. However, the was accompanied by negative changes to their texture and juiciness [20]. These observations are in agreement with the previously reported changes in dry cured loin immediately after the PL treatment of 11.9 J/cm<sup>2</sup>, when odor and flavor also significantly decreased [22].

Tomasevic [20] also noted that the sensory quality of 1-pulse treated fermented sausage was not significantly different to the untreated fermented sausage, which is in concurrence with the findings of Ganan *et al.* [22] where also no significant differences were observed in salchichón treated with different fluences. When the fermented sausage was exposed to a 5-pulse treatment, its temperature raised by 12°C and the texture and juiciness was significantly affected. In another investigation of Tomasevic [23], all of the seafood samples were assessed as very acceptable, with the total score value greater than

4.5, no matter if they were PL treated or not. Even though significant changes in odor were assessed after the 5-pulse treatment, it was still described as pleasant and acceptable. Tomasevic [23] could not confirm the development of sulfur notes in tuna induced by the fluences higher than  $8.4 \text{ J/cm}^2$ , as Hierro *et al.* did [19]. All the other sensory attributes evaluated remained unaffected by the PL treatments [23]. Ozer and Demirci [24] also noticed that PL treatment of  $5.6 \text{ J/cm}^2$  did not affect the quality of salmon fillets.

The latest study carried out by Koch *et al.* [16] associated the most intense PL treatments ( $4.96$  and  $12.81 \text{ J/cm}^2$ ) with unpleasant, ozoneous, pungent, ammoniacal, and off-odor perception in pork skin and loin. Conversely, the samples subjected to  $0.52 \text{ J/cm}^2$  were perceived as “less porky” and “slightly chemical”, which supports the indications of Tomasevic [18,20] that excessive PL treatment can seriously affect the sensory quality of certain types of meat and meat products.

## 5. Instrumental color measurements

Tomasevic [18] found the instrumental color values of beef meat were not affected by 1-pulse treatment. Treatment of 5 pulses significantly decreased redness in beef, while no significant differences were observed for lightness and yellowness. The changes in redness, although significant, were not sufficient to be noted by the sensory panel [18]. In beef carpaccio subjected to PL, Hierro *et al.* [19] also observed decreases in  $a^*$  (redness) values but they were accompanied by significant differences in  $b^*$  (yellowness) value when the samples were treated with fluences equal to or higher than  $8.4 \text{ J/cm}^2$ . Tomasevic reported [18] that pork  $a^*$  and  $b^*$  values significantly decreased after the treatment of  $17 \text{ J/cm}^2$ , while chicken color values were not significantly changed, irrespective of the level of treatment. This is in agreement with the results of Keklik *et al.* [25], also indicating that mild and moderate pulsed light treatments did not affect the color of chicken samples, although extreme PL treatment did increase the lightness ( $L^*$ ),  $a^*$ , and  $b^*$  values of samples significantly. The  $a^*$  value of treated turkey samples were significantly lower than that of the untreated samples with significant differences observed among the fluences assayed by Tomasevic [20]. The redness gradually decreased as fluence increased. Similar PL color resistances as were observed in chicken meat were noted only in rabbit meat samples. Venison suffered significant decrease in  $a^*$  value after 5-pulse treatment, while kangaroo meat had significantly lower  $L^*$  (after 1-pulse) and  $b^*$  (after 5 pulses) [18].

In the investigation of Tomasevic [20], PL lightened the color of cooked ham after the  $17 \text{ J/cm}^2$  treatment was applied. The  $a^*$  value gradually decreased as fluence increased, while only the highest fluences significantly affected the  $b^*$  value, similar to observations by Hierro *et al.* [21]. The lightness of Parisian sausage remained unaffected during the Tomasevic [20] investigation, while redness and yellowness suffered significantly, with observed differences among the fluences assayed. The significant increase in  $b^*$  values of cooked ham after PL treatment was previously reported [26], as it was in other cooked-meat products like bologna [21] and chicken frankfurters [14].

As reported by Tomasevic [20], Parma ham  $L^*$  was significantly lower in ham treated with  $17 \text{ J/cm}^2$  compared to control and ham treated with  $3.4 \text{ J/cm}^2$ , while in fermented sausage and bacon,  $L^*$  remained unaffected by PL. The lightness of dry-cured loin also endured, while it was significantly higher in salchichón (fermented sausage) treated with  $11.9 \text{ J/cm}^2$  as reported by Ganan *et al.* [22]. The  $a^*$  value significantly decreased after the 5-pulse treatment in Parma ham, fermented sausage and bacon, while the  $b^*$  value significantly increased only in bacon [20]. It has been reported that when cured meat products are exposed to light, discoloration appears as a decrease in  $a^*$  values and an increase in  $b^*$  values, with or without a change in  $L^*$  [27].

Pulsed light darkened tuna when examined by Tomasevic [23], but only after the fluence of  $17 \text{ J/cm}^2$  was applied, while  $a^*$  and  $b^*$  values were not significantly different to the control. This is contradictory to [19], where  $8.4 \text{ J/cm}^2$  significantly increased  $L^*$  and decreased  $a^*$  and  $b^*$  values in tuna carpaccio. The lower dose of  $3.4 \text{ J/cm}^2$  significantly affected none of the color values in tuna [23], similar to the results of Figueroa-García *et al.* [28] for catfish and of Cheigh *et al.* [29] for flatfish where no changes were observed in the CIE  $L^*$ ,  $a^*$  and  $b^*$  at fluences lower than  $2 \text{ J/cm}^2$ . The color of flounder and crab was not significantly affected [23] by PL.

## 6. Conclusion

PL increases the shelf life of meat, fish and derivate products as well as achieving decontaminating effects on meat contact surfaces. In general, around 2.0 log CFU/mL reduction of the initial count of microorganisms was achieved after PL treatments, independently of the target microorganism and the matrix. However, it is still necessary to optimize the treatment conditions and take into account that the effectiveness of PL depends on the time among contamination, PL treatment parameters and food matrix. According to the evidence provided so far, it seems convincing that because of the short duration of the pulse, PL does not encourage oxidative changes in lipids.

The meat sensory quality changes induced by PL are varied and depend on animal species, type of meat and PL dose applied. Instrumental color values remained unaffected in chicken and rabbit meat while higher doses of PL significantly compromised both redness and yellowness only in pork and turkey meat. The sensory quality changes induced in meat products by PL are also varied and depend on type of meat product and PL dose applied. PL caused minimum changes in the sensory properties and instrumental color values of dry cured and fermented meat products. There is a lot of potential for the commercial application of PL for the decontamination of seafood products because the sensory quality of seafood induced by PL is almost unaffected and independent of type of seafood and PL dose applied. Only the odor of the majority of investigated seafood samples suffered significant changes after PL treatment.

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# Qualitative properties of traditionally produced dry fermented sausages from meat of the autochthonous Mangalitsa pig breed

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**Abstract.** The interest in autochthonous meat products from local pig breeds managed in comprehensive, sustainable breeding programs is expanding in Europe. Dry fermented sausages in Serbia and other southern European countries are highly appreciated food specialties. It is, therefore, desirable that study attempts to improve the quality of food and the security of traditional, dry sausages will result in products that are of higher added value and have quality standards that best meet the needs of contemporary customers. Meat and meat products from traditional pig breeds usually have an excellent public and media reputation, and are often regarded as better than the meat and meat products of conventionally raised pigs and crossbred pigs. Traditional, dry fermented sausages, with their characteristic chemical contents and sensory properties, can be produced with suitable proportions of meat and backfat from the indigenous Mangalitsa pig breed. These outcomes should hopefully encourage the sustainable breeding of endangered Mangalitsa pigs, as there are market opportunities for *kulen* and *sremska* sausages.

## 1. Introduction

Traditional food is a significant part of European culture, identity and heritage [1] and is also strongly accepted among Serbian consumers [2]. Serbian culture is deeply rooted in a living tradition in the production of meat and meat products. Serbia is a relatively small country, but it contains numerous institutions for the production of meat and meat products [3]. Most meat establishments in the country are small enterprises that matter to the local society or region in which they are situated. Pork is traditionally the most commonly consumed meat species, correlating with pork product production, which are by far the most commonly manufactured meats [2]. In some parts of the country, beef is also highly valued and consumed more commonly, especially for religious reasons [7].

The typical gastronomic specialties in Serbia are a number of dry fermented sausages (locally known as *kulen* and *sremska*). These products, often from indigenous pig breeds, have increasing popularity as part of traditional Mediterranean foods and are increasingly consumed by Serbs and visitors alike during leisure or social occasions, when they are eaten together with other foods such as regional cheeses, bread and wine, or provided as an opportunity to discover traditional cuisine. This trend thus anticipates important possibilities for the development of these products on the market and the evolution of restaurants, snack bars or taverns, both gourmet and regional. In contrast, and in parallel, consumers are advised to reduce their daily meat consumption, including that of dry fermented sausages and meat product equivalents. This



advice can be ambiguous and it seems possible it could have damaging consequences for the meat industry and the meat market.

The interest in indigenous meat products produced from local pig breeds in extensive, sustainable breeding programs in Europe, especially in Mediterranean countries, is on the increase. Meat and meat products from traditional pig breeds are typically well-regarded by the public and media, and often are better-regarded than modern pig and crossbreed meat and meat products. A suitable combination of meat and fat from indigenous pig breeds can be used to produce traditional dried fermented sausage with authentic chemical contents and sensory characteristics. These should contribute to the maintenance of autochthonous pig breeds, provided there are market opportunities for traditional dry fermented sausage.

## **2. History and current status of the autochthonous pig breeds**

Animal genetic resources in pig-breeding in Serbia include three breeds: Mangalitsa, Moravka and Resavka. Mangalitsa is a fatty breed, while the other two have combined production abilities (they are fatty-meat breeds). In the last thirty years, the populations of autochthonous breeds have been reduced; there was a mating in kinship and deterioration in the production performance of these breeds. Moravka and Resavka are critically endangered pig breeds and there is a real chance they will become extinct. It should be pointed out that there are efforts to increase the number of autochthonous breeds' populations, which are recognised by the relevant ministry.

Mangalitsa is an autochthonous fatty pig breed of the old Šumadinka breed of Serbia. Pigs were Serbia's main export product during the nineteenth century, particularly in the northern part of the country (today's Autonomous Province of Vojvodina) and in the region of Šumadija (central part of Serbia). Pigs were mostly fattened in the forests in Šumadija, where they consumed oak and beech acorns and other forest feed resources. In Serbia, there are three Mangalitsa breed types: *swallow-belly*, *white* and *red*. Mangalitsa is present in Germany, Austria, Hungary, Slovakia, Romania and Switzerland, as well as in Serbia. At the end of 2017, approximately 67 farmhouses with 925 sows, 605 gilts and 42 boars, of which more than 95% were swallow-belly, were registered across the country by the main breeding organisation in Serbia.

## **3. Geographical location and production system of Mangalitsa pigs**

Farms holding Mangalitsa pigs in Serbia are in the municipalities of Subotica, Sremska Mitrovica, Bačka Palanka, Vršac, Pančevo, Ub, Obrenovac, Ljig, Valjevo, Novi Sad, Kuzmin, Šid, Surčin and Kovilj (Krčedinaska ada). There are also some Mangalitsa pigs on Stara Planina Mountain (Dimitrovgrad Municipality) and around Čačak and Kraljevo. These pigs are mainly bred in free-range outdoor, extensive environments, or semi-intensive production systems. This type of pig rearing means the animals free-range on pastures within restricted fields, or in woods or orchards. Pigs can range around the community in an extensive system, which depends on the number of livestock and the size of the owner's estate, and animals are transferred into cheap and effective wooden housing during the winter. Feeding is based mainly on pasture forage and forest edibles (acorns and wild fruits). An additional daily meal is an extremely small quantity of grains per head, mainly maize. In the extensive system, sows frequently farrow in the forest, which significantly complicates productivity control and recording. Sows are also farrowed in housing under semi-intensive conditions, enabling better control. The pigs are mostly outside in the growing and fattening phases.

## **4. Meat quality of Mangalitsa pigs**

Table 1 presents the basic data obtained for some common meat and fat quality characteristics measured in the *longissimus* muscle from Mangalitsa pigs. In publications presenting the meat quality of Mangalitsa pigs, the pH measured in the *longissimus* muscle was around 6.1 [18, 19, 20, 21, 22] and 5.6 [18, 23, 19, 24, 21, 22, 25]. In reported studies, the intramuscular fat content ranges from 2.9% to 18.2% [23, 27, 20, 21, 22, 24, 25, 28, 29, and 31]. The colour measured in CIE L, a, b colour space was approximately 45, 11.4 and 4.2 for L, a\* and b\*, respectively [18, 23, 19, 25], indicating a relatively dark colour of Mangalitsa meat. In the considered studies, the intra-muscular fat contents of saturated

fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were about 35.5% 55.4% and 7.0%, respectively, with high n6/n3 ratios (9.2-37.3) [27, 19, 24, 21, 32].

**Table 1.** Summary of the meat quality content recorded in the Mangalitsa pig breed.

Reference	No. of animals	pH45	pH 24	CIE <sup>1</sup>			IMF content (%)	Fatty acid composition <sup>2</sup> (%)			
				L*	a*	b*		SFA	MUFA	PUFA	n-6/n-3
[18]	35	5.95	5.77	56	10.3	5.1	—	—	—	—	—
[23]	15	—	5.46	46	12.8	5.2	8.4	—	—	—	—
[27]	16	—	—	—	—	—	5.1	39.5	56.4	4.1	—
[19]	12	6.11	5.50	40	11.8	3.7	—	33.3	50.3	11.6	17.9
[28]	10	—	—	—	—	—	13.2	—	—	—	—
[20]	—	6.04	—	—	—	—	8.1	—	—	—	—
	—	6.32	—	—	—	—	5.5	—	—	—	—
[24]	12	6.12	5.80	—	—	—	18.2	33.9	57.2	5.9	37.3
	10	5.89	5.41	—	—	—	12.1	35.5	55.5	6.5	9.2
[21]	24	6.01	5.68	—	—	—	15.2	34.6	56.6	6.1	14.1
[29]	16	—	—	—	—	—	9.8	—	—	—	—
[22]	—	6.42	5.56	—	—	—	2.9	—	—	—	—
[25]	7	—	5.47	38	10.9	2.9	6.4	—	—	—	—
[32]	22	—	—	—	—	—	—	35.6	56.6	6.9	25.1

No. = number, pH 45= pH recorded after estimated 45 minutes, pH 24= pH measured post rigor estimated 24 hours, IMF= intramuscular fat, SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids. <sup>1</sup>CIE= objective colour defined by the Commission Internationale de l'Eclairage; L\* higher value refers to a lighter colour; a\* higher value refers to a redder colour; b\* higher value refers to a more yellow colour. <sup>2</sup>For the compositions of fatty acids, only dietary control pigs have been considered. Control diets varied between studies to determine appropriate diet composition.

## 5. Use of breed and main products

Mangalitsa pigs are late maturing and are chosen for fat production. The breed has low fertility, lengthy sucking times, and very slow growth. On the other hand, however, Mangalitsa pigs are very hardy and well adapted to extensive housing conditions, where only a simple shelter from rain and snow is required. Their cost effectiveness is on par with such features, in the context of low investment in housing facilities, but large areas required for pasture and acorn feeding. Due to low production performance (low daily gain and carcass live weight), cross breeding with the Moravka, Resavka, Duroc, Hampshire or Berkshire breeds could help improve growth and carcass traits while reducing the fattening period and increasing the meat content of the carcass. Radović et al. [19] showed not significantly improved growth rates between Mangalitsa and Mangalitsa × Moravka crossbreeds (average daily gain, 267.9 vs. 336.9 g) and not any less carcass meat (33.2% vs. 33.9%). Animals not selected for the nucleus herd could be crossed with Duroc, Hampshire or Berkshire to help produce more economical meat and high-value products in the traditional style (ham and *kulen* and *sremska* sausages) that could be marketed as highly valuable organic products or geographically protected products. Dry fermented sausages are long-established meat products, and today there are numerous national varieties. *Kulen* [33, 34] and *sremska* sausage are the most common types of traditional dry fermented sausages in Serbia. Figure 1 shows the main traditional *kulen* and *sremska* dry fermented sausage production processes.

Processing steps	Raw material and ingredients	Technological parameters	Checking measures
↓	↓	↓	↓
Slaughtering	Heavy pigs (>120 kg)		
↓	↓	↓	↓
Selection of carcasses, meat and back fat	Leg (I cat. meat) Back (I cat. meat) Shoulder (II cat. meat) Neck (III cat. meat) Firm back fat	Carcass cooling, 24 h	
↓	↓	↓	↓
Meat cooling and drainage		Temperature around 5°C, 12-24 h	
↓	↓	↓	↓
Raw meat preparing for production sausages		Lean meat cleaned from fat and connective tissue	Raw meat of good quality; Rapid chilling of meat; Meat examination for <i>Trichinella</i>
↓	↓	↓	↓
Meat chopping, mincing and mixing	+ Ratio of I:II or II+III meat category = 75:25 + Back fat + Salt + Hot red paprika + Mild red paprika + Garlic	Storage of spices in dry space; Minimum table salt added 2.2%	
↓	↓	↓	↓
Stuffing into natural casings and binding		Cleaned and washed pork cecum, binding with hemp rope	Adequate preparation of natural casings
↓	↓	↓	↓
Fermentation and smoking		Natural fermentation, temperature 8 - 25 °C, 30 day	Control of smoking conditions and optimal smoking temperatures and humidity
↓	↓	↓	↓
Drying and ripening		Temperature 10 -15 °C, 150 - 180 days, weight loss 40 - 50%	Temperature and humidity control; Control of the pH
↓	↓	↓	↓
Storage		Cool, dry and dark place	

**Figure 1.** Traditional *kulen* and *sremska* dry fermented sausage production processes

## 6. Kulen – traditional dry fermented sausage

*Kulen* is a well-known and popular traditional dry fermented sausage in Northern Serbia (Srem, Bačka) and Croatia (Slavonija, Baranja). For all variants of this product, high-quality meat is

used from mature pigs with relatively low water content, intense red colour and firm consistency. The meat used is mainly leg, shoulder and some neck pieces, but also with a small percentage of firm backfat tissue. Muscle and adipose tissue (75:25) are typically cut to 10 mm granulation in a cutter. The chopped meat is transferred to a mixer and the other filling ingredients are added: 2.3 % table salt, 0.4 % saccharose, 0.3 % garlic (powder), 0.3 % pepper and 0.8 % ground sweet and hot red paprika. Then the filling is firmly stuffed into natural pig colon casings. Sausages are hung on rods and left 20 to 24 h at 18 °C for the surface to dry. After that, sausages are moved to a traditional smokehouse. Sausage production, smoking and maturation occur during winter (December to February). The temperature of the smokehouse is from 10 to 15 °C and the humidity is between 75 and 90%. Subsequently, sausages are matured at 10 to 12 °C and in industrial conditions, in a controlled drying chamber until the end of the manufacturing process, which lasts 90 days.

### 7. Sremska – traditional dry fermented sausage

Sremska sausage is a traditionally produced Serbian dry fermented sausage from the northwest part of Serbia (Srem region), where it was produced in village households. It is made from ground pig meat and backfat (approximately 8 mm) and mixed with salt and spices. *Sremska* sausage has a pronounced red colour, tender texture, slightly hot taste, fermented meat and mild spice and smoke notes [35, 36].

*Sremska* sausage was traditionally manufactured in smokehouses during winter [35]. The manufacturing technology for most dry fermented sausages is now based on modern technology, controlled maturing rooms and fast cure methods, leading to reduced manufacturing time and safer product [4, 5]. Industrial *sremska* sausages acquire exceptional appearance characteristics, but typically, their other sensory attributes are poor. They have, above all, a strong acidic flavour that is largely unacceptable to consumers [7]. On the other hand, traditional *sremska* dry fermented sausages manufactured at low temperatures by spontaneous meat fermentation are of very high quality [31].

For industrial manufacture of *sremska* sausage, shoulder meat and backfat from Mangalitsa pigs (approximate live weight 115-120 kg) is minced in the ratio of 75:25 then blended in a cutter. The cut meat/backfat is blended with other components: 2.2% NaCl, 0.3% sugar, 0.17% garlic (powder), 0.55% hot red paprika (powder) and 0.55% sweet paprika (powder). No starter culture is added, so fermentation is spontaneous. The sausage filling (approx. 700-800 g) is stuffed into natural casings of about 32 mm in diameter (pig small intestines). Sausages are held in a cold store (4±1 °C) for 12 hours for their surfaces to dry and then placed in a traditional smokehouse. The ripening is as follows: the first stage lasts 14 days in a traditional smokehouse at 10-15 °C with 75-90 % relative humidity (RH), where the sausages are smoked for 6 hours each day; during the next 7 days, sausages are processed in a drying room at 14-16 °C with about 75 % RH, reaching about 35.0 % humidity. The complete processing time is 21 days.

Current knowledge of traditionally manufactured *sremska* sausage is restricted and their quality is very variable as there is very little uniformity in the manufacturing practices applied by distinct meat and home manufacturers. Within the current trends of encouraging and supporting successful traditional food manufacturing technologies and in order to maintain the quality of traditional *sremska* sausage, the physico-chemical qualities of this sausage manufactured in a traditional smokehouse have been studied.

### 8. Quality properties of *kulen* and *sremska* dry fermented sausages

Tables 2 and 3 present the basic physico-chemical properties of *kulen* and *sremska* dry fermented sausages, respectively.

**Table 2.** Physico-chemical properties of *kulen* traditional dry fermented sausage

Reference	Moisture	Protein	Fat	Ash	pH	Fatty acid composition (%)				Cholesterol
						SFA	PUFA	MUFA	n-6/n-3	
	%	%	%	%						mg/100 g

[37]	27.58	24.14	43.36	4.09	-	39.10	11.57	49.34	25.19	62.07
[34]	-	33.75	20.86	5.37	-	39.77	14.07	46.16	-	68.14
[38]	37.8	31.0	24.2	-	5.22	-	-	-	-	-
[33]	38.06	34.62	16.98	5.34	5.12	39.00	12.92	48.44	22.05	68.64
	37.92	35.79	15.02	5.25	5.05	42.21	13.49	45.77	18.87	61.48
	35.70	34.24	21.00	5.67	5.03	40.72	13.46	46.19	22.25	66.00
	37.55	35.63	14.98	5.91	4.99	40.44	12.93	46.99	23.02	64.76
	38.78	35.04	17.10	5.46	4.86	40.29	14.80	45.47	17.57	79.62
[39]	23.62	27.20	39.30	4.61	5.24	39.22	8.80	51.97	16.96	50.16

SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

**Table 3.** Physico-chemical properties of *sremska* traditional dry fermented sausage

Reference	Moisture	Protein	Fat	Ash	pH	Fatty acid composition (%)				Cholesterol
						SFA	PUFA	MUFA	n-6/n-3	
	%	%	%	%						mg/100 g
[36]	27.89	21.46	44.78	6.24	5.48	-	-	-	-	-
	25.11	23.09	44.98	6.05	5.50	-	-	-	-	-
[37]	28.17	22.04	43.83	5.08	-	37.19	12.07	50.74	26.39	60.72
[39]	21.67	29.16	39.45	5.25	5.25	38.40	8.78	52.80	14.38	59.65
	39.41	28.04	22.00	4.72	4.72	39.71	16.78	43.49	35.86	64.92
	33.30	23.20	34.92	4.73	4.73	40.94	14.00	45.04	37.36	53.47

SFA= saturated fatty acids, MUFA= monounsaturated fatty acids, PUFA= polyunsaturated fatty acids.

### 8.1 Basic Chemical Composition

The protein content of *sremska* sausage was 21.46% to 29.16% [36, 37, 39]. The highest protein content in *kulen* sausage was 35.79% [33]. *Kulen* and *sremska* sausages made from the meat of Mangalitsa pigs had the lowest moisture content (21.67% and 23.62%, respectively), and the highest fat content (44.98% and 43.46%, respectively) among other sausages from other pig breeds. Some studies have already shown that Mangalitsa meat (*m. longissimus*) has a reduced protein content compared to the meat of commercial pig breeds [30, 40], thus explaining in part the moderately reduced protein content in *kulen* and *sremska* sausages. The low moisture content is typical of similar products from Greece, Hungary and Croatia [41], and is a consequence not only of drying, but also the relatively high fat content. The ash contents of the *kulen* and *sremska* sausages varied, ranging from 4.09% to 6.24%. The pH of *sremska* sausages ranged from 4.72 to 5.50 [39]. At the beginning of the ripening process of traditional *kulen*, the pH ranged from 5.6 to 5.8, which corresponds to the pH of cooled pork meat, and subsequently, the sausage pH starts to decline [42]. Certainly, the sugars (fructose, glucose, sucrose) that are the paprika spice's natural components have a major impact on the pH value of maturing *kulen*. According to literature data [43], ground paprika contains about 15% sugar, whereas the complete sugar content of local ground paprika is greater and about 25%.

In Italy [44, 45], Greece [46], Spain [6] and France [8], the naturally dry fermented sausages from the Mediterranean countries are usually characterised by low acidity with a final pH range from 5.2 to 6.4.



### 8.2 Fatty Acid Composition

Tables 2 and 3 show the fatty acid profiles of *kulen* and *sremska* sausages. The levels of PUFA in other dry fermented sausages made from the Mangalitsa pig breed were around 8.78 and 8.80% and 16.78 and 14.80%, respectively [33,39]. Lower complete n-3 PUFA content and reduced levels of total n-6 PUFA generated these distinctions. These led to lower n-6/n-3 ratios in dry fermented *kulen* (17.57) and *sremska* (14.38) sausages. In spite of that though, the n-6/n-3 ratios in other types of sausages were between 25 and 37. In different studies, Hoz [9] and Valencia et al. [10] found reduced ratios of n-6/n-3 fatty acids (12.05 and 13.86, respectively) in their control groups of dry fermented sausages. MUFA values ranged from 43.49/45.47% to 52.80/51.97%. The *sremska* and *kulen* sausages made from the meat of Mangalitsa pigs contain higher levels of MUFA than other types of sausages. Additionally, oleic acid (C18:1 cis-9), cis-vaccenic acid, (C18:1 cis-11) and palmitic acid (C16:1) levels in these types of sausages were considerably higher than in the other types [39]. *Kulen* and *sremska* sausages made from the meat of Mangalitsa pig breed have higher unsaturated fatty acids (USFA) and lower SFA levels. Overall, USFA contents are significantly higher in sausages made from the meat of Mangalitsa pig breeds [39].

### 8.3 Cholesterol Content

The cholesterol content in *kulen* and *sremska* sausages at the conclusion of the production process ranges from 53.47/50.16 mg/100 g to 64.92/79.62 mg/100 g [33, 39]. Cholesterol levels have been established between 94.8 and 110.5 mg per 100 g for Salami Milano [11]. For Italian salami, cholesterol contents of between 48 mg and 57 mg/100 g were measured by Baggio and Bragagnolo [12]. Pleadin et al. [13] noticed that the average cholesterol content was from 58.48 mg/100 g to 105.24 mg/100 g in sausages that were industrially prepared, while in home-made sausages, the cholesterol content reached 75.07 mg/100 g.

### 8.4 Sensory Properties

*Kulen* and *sremska* sausage colour is correlated with the colour of the meat used. Mangalitsa pig meat is darker than other pork (for example, Swedish Landrace and Moravka); therefore, sausages made from the meat of Mangalitsa breed were assessed as too dark, and received a somewhat lower grade than the other sausage types [39]. Odour was the sensory indicator most affected by the pig breed. The most characteristic and finest sausages are made from Mangalitsa breed meat. The odour of this sausage type was rich and very pronounced, and received a much higher grade than other kinds of sausages [39]. Sausages made only from the meat of Mangalitsa breed (*kulen* and *sremska*) had better sensory characteristics, thus confirming the work of Radman et al. [14], who observed some pig breeds are appropriate for dry fermented pork sausage manufacture. Relationships have been reported between physical meat quality characteristics and sensory characteristics, such as muscle fibre and overall tenderness [15, 16], and between quantity and composition of intramuscular fat and flavour [26]. Flavour is, however, a very sophisticated attribute of meat palatability [26] and its relation to fat content and structure varies with cattle breed [16].

## 9. Sustainable development: economic, environmental and social points of view

Serb consumers greatly appreciate the meat, adipose tissue and meat products from Mangalitsa pigs, and scientific effort is not limited to just preserve the breed as such, but also to exploit the animal's potential for human consumption. The production of these pork specialties plays important roles based on the three pillars of sustainable development: economic, environmental and social. The utilisation of all parts of livestock animals, with minimal losses and food waste, is of great financial and environmental significance. This sustainable strategy also helps to provide farmers with extra revenues in an economic and environmental sense. In addition, indigenous livestock production is a significant financial activity in the eastern and western regions of Serbia (where the main indigenous pig industry remains crucial in gross domestic product (GDP) terms), although it also exists at a smaller level throughout the country. The various meat companies and their extension businesses also help to attract individuals to live in and



remain in rural communities and areas, thereby stopping the current rural exodus to metropolitan areas and helping to promote (now booming) local tourism.

Food safety and quality are major concerns and European Union (EU) policy priorities, as highlighted earlier in the White Paper on Food Safety, Agenda 2000, as well as in Horizon 2020 – the EU Research and Innovation Framework Programme. The relevant knowledge on science-based features of these sausages will, therefore, be submitted to formal accreditation focused on traditional products through Protected Geographical Indication (PGI). This will contribute to improving sausage quality and to rationalising any health claims more effectively, with both helping to expand the market niche and the economic value of the foods [17].

Every effort to enhance the quality and safety of foods like traditional dry fermented sausages and other products is always worthy. Accordingly, scientifically sound work on defining traditional dry fermented sausages derived from a wide range of technological studies (rigorously designed and on products developed to be implemented in the meat sector) and in cooperation with the numerous regional meat producers and suppliers is essential. For example, the use of different salt levels, various ingredients and raw materials, change of sausage diameter, smoke type and duration, and animal genotype are examples of relevant technological parameters than need to be assessed throughout the manufacturing process (for example at start of processing, during maturation, in final product and during distinct storage stages). Maturation times and temperatures and the use of suitable preservative compounds (e.g. acetic or lactic acids) are worthy of study.

This type of scientific effort by the research community is expected to result in a significant improvement in the quality of dry fermented sausages and other meat products, and thus should contribute to market expansion of these products [17]. This will in turn lead to numerous beneficial effects such as enhancing South European and Mediterranean foods and their quality, preventing rural exodus to urban areas and enhancing the economy as a whole – upstream (stimulating autochthonous pig breeds) and downstream (incitement to the meat industry).

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## Novel paradigms linking salt and health

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**Abstract.** Although sodium is an essential nutrient, conclusive scientific evidence suggests the association between excessive salt intake and various negative health outcomes. One of the health consequences with the greatest public health impact is the increase in population blood pressure with a consequent increase of cardiovascular disease risk. There is ample evidence linking high salt intake with other health outcomes: stomach cancer, impaired renal function, osteoporosis, obesity, severity of asthma, but also with novel health risks established with advanced molecular and metagenomics technology: autoimmunity, immunity in various organs and systems. Some recent studies have reported that a high salt diet modulates the gut-microbiome, interacting with both the host's gastrointestinal tract environment and its genome and metabolism. The newest evidence indicates possible novel pathophysiological mechanisms of obesity, including high fasting ghrelin in healthy individuals consuming a high-salt diet, as well as endogenous fructose production and leptin resistance in mice. This revealing new evidence links high salt intake with obesity and consequently, with further metabolic complications. As a country with high prevalences of obesity and hypertension, and high salt intake, Serbia would greatly benefit from adopting and implementing a national sodium reduction program that minimize risks through education, regulation, and enforcement.

### 1. Sodium

Sodium is an essential nutrient for the physiology of the human body, interrelated with potassium, playing vital roles to maintain physiological homeostasis. It is the principal extracellular cation, corresponding to the leading intracellular cation, potassium (K<sup>+</sup>). Their interaction generates transmembrane electrochemical potentials, fundamental for electrical signaling in the nervous system, muscle, and heart. One of the most important roles of sodium is its involvement in extracellular fluid volume control and systemic distribution of total body water, and thus in blood pressure (BP) regulation. The amount of sodium required for all these demanding physiological roles is very small [1]. Sodium transport and co-transport systems of water and solutes are energy demanding processes consuming around 25% of the resting metabolic rate [2]. There is also new evidence suggesting that sodium metabolism is closely related to energy metabolism, linking high salt intake with non-



communicable chronic diseases (NCD's) such as obesity, diabetes mellitus, osteoporosis, and increased cardiovascular and neurovascular disease risk [3].

Blood concentration of both cations is tightly regulated by multiple homeostatic mechanisms maintaining plasma sodium and potassium concentrations of healthy individuals within a narrow range [4]. During the process of evolution, *Homo sapiens* has developed a high survival capacity in extreme sodium intake ranges, developing adaptive physiological mechanisms that reduce the losses of sodium in urine, feces, and sweat, and maintain blood sodium concentrations at low levels [5]. In the contemporary food environment, the dietary sodium intake from multiple sources far exceeds the physiological minimum and puts pressure on the homeostatic mechanisms. Novel evidence suggests the existence of biological clock in sodium and potassium kinetics, a circadian rhythm of urinary excretion of sodium and potassium, affecting water balance, glomerular perfusion, and filtration rate, and BP [4,5,6].

The nephron in the kidney is the leading “organ” regulating retention and excretion of sodium and, therefore, water homeostasis. In conditions of low dietary sodium intake, it efficiently saves sodium, and, conversely, in response to high dietary intakes, excretes the excess sodium [7]. Almost all ingested sodium (92.8%) is excreted in urine (ranging from 76% to 122% across studies), so 24-h urine collection is appropriate to measure dietary sodium intake [8, 9]. After the kidneys filter out all dietary sodium, more than 99% is reabsorbed in the renal tubules [7]. The number of nephrons and the glomerular filtration rate decreases with age, which reduces the efficiency of the regulatory system and partially explains the physiological occurrence of increasing BP with age in populations with high sodium intake [10]. While the loss of sodium *via* feces is relatively stable, losses *via* sweat vary widely and can be considerably higher in situations of extreme exercise and heat [11]. After excessive sodium intake, renal sodium excretion is established after about 24 h. A state of balance between sodium intake and urinary sodium excretion is considered to be achieved within a few days [12].

## 2. Dietary salt

The leading source of sodium in human nutrition is dietary salt, or sodium chloride (NaCl). One gram of sodium chloride provides 0.4 g of sodium and 0.6 g of chloride (17 mmol sodium and chloride) [2]. Humans are genetically predetermined to eat a diet with small quantities of salt, mainly naturally present in animal and plant food [13]. The role of salt in food preservation led to its long and very influential role in the history of humanity. Since the dawn of civilization, it has been one of the key factors in economic, social, spiritual, religious and political development. Salt is still highly valued, because of its microbiological, technological and sensory significance, primarily in food preservation [14].

Western diets are characterized by a high dietary intake of processed food, and even more, with ultra-processed food. When used in small quantities, those products are harmless. However, processed foods have intense taste and palatability, and are subject to sophisticated and aggressive marketing (reduced price for super-sized serving, free refill, social media). These factors contribute significantly to high intake of energy, fat, saturated fat, added sugar, additives, and salt, along with low fiber, vitamin, and mineral intake on consuming processed foods [15]. Epidemiological studies have found associations between consumption of processed food and the risk of cardiovascular diseases (CVD), a higher risk of dyslipidemia and higher incidences of overweight and obesity, as well as a higher risk of overall cancer and breast cancer [16].

High dietary salt intake consumed with processed foods or by adding salt during cooking or at the table, has long been associated with high BP, a major risk factor for CVD and cerebrovascular diseases [17]. New evidence also connects high dietary salt intake with other health risks [18].

### 2.1. Dietary salt intake

Dietary sodium/salt intake can be measured using different methods (2.5g of salt contains 1g of sodium) [9]. Blood sodium concentration is not a reliable indicator of usual dietary sodium intake

reflecting most often inadequate water balance, rather than salt intake. A number of different ways of measuring dietary sodium (salt) intake are currently available, including dietary (24-h dietary recall, Food Frequency Questionnaires, diet records) and urinary assessment (24-h urinary sodium excretion, spot urine). Selection of the appropriate method must be in accordance with the research interests, the profile of the respondents and their environment, and available resources. Population sodium intake should be a valid estimate of the range and frequency of dietary salt intake across the population, and must provide a valid estimate of mean population level intake in the representative population sample [19]. The World Health Organization (WHO) recognized 24-h urine collection as a standard method appropriate to measure dietary sodium intake [20, 21]. Sodium intake assessed by dietary records could lead to underreporting of salt intake by 29-41% [22].

One of the biggest systematic reviews, conducted as part of the 2010 Global Burden of Diseases (GBD), Injuries and Risk Factors Study, estimated the mean level of sodium consumption during 2010 in 187 nations (covering almost 74.1% of adults worldwide) was 3.95 g per day (corresponding to 9.88 g of salt), and regional mean levels ranged from 2.18 to 5.51 g per day. Estimated sodium intake level is nearly twice the WHO recommended reference intake of 2.0 g of sodium per day (5g of salt per person per day) [23; 24]. Similarly, according to data collected by the European Commission, salt intake in European adults ranges from 7 to 13 g per day [25]. While a national study of population salt intake has not yet been carried out in Serbia, salt intake measured by the reference 24-h urinary sodium excretion method was reported in Novi Sad. Average salt intake of 12.12 g per person per day was measured [26], similar to other countries in the sub-region, like Montenegro with an average salt intake of 11.6 g/day [27], and Slovenia with 11.3 g/day [28].

## 2.2. *Dietary sources of salt*

The leading dietary source of salt/sodium in developed countries, about 75-80%, is processed food, and 5-10% is obtained from sodium naturally present in the foods. These are non-discretionary salt sources that occur inherently in food or are due to salt being added during food production, manufacture or processing. The remaining 10-15% comes from salt added during cooking or at the table [29, 30]. In some countries, like many Eastern countries, the predominant source of salt in food is discretionary salt, from added condiments (soy sauce) to food, comprising 76% of total salt intake [22].

Despite some regional differences, in most European countries, four food categories are the main contributors to salt intake: bread, cereals and bakery products as a staple food, then meat and meat products, cheese and dairy products and canned and pickled vegetables [22]. Similar results were found in a study on the student population in Novi Sad, Serbia [31]. Those food groups are also targeted for reformulation in most countries where salt reduction actions occur.

## 3. **Salt and health**

Although sodium is an essential nutrient, conclusive scientific evidence systematically reviewed by leading health authorities suggest the association between excessive salt intake and various negative health outcomes [1,21,32]. Epidemiological data show that in 2017, more than half (3 million) of diet-related deaths and two-thirds (70 million) of diet-related DALYs (disability adjusted life years) globally were attributable to sodium intake greater than 3g per day (7.5g of salt per day), the leading dietary risk factors for deaths and DALYs globally [33].

One of the health consequences with the greatest public health impact is the increase in population BP with a consequent increase of CVD risk [17]. There is numerous evidence linking high salt intake with other health outcomes: stomach cancer, impaired renal function, osteoporosis, obesity, severity of asthma, but also with novel health risks established with advanced molecular and metagenomics technology: autoimmunity, and immunity in various organs and systems (brain, kidney, skin, and vasculature) [18]. Some studies have reported that high salt diet modulates the gut-microbiome interacting with both the host's gastrointestinal tract environment and its genome and metabolism,

revealing new evidence linking high salt intake and obesity, one of the major public health concerns, and consequently, with further metabolic complications [34].

### 3.1. Salt and cardiovascular diseases

It is indisputable that high salt intake is a determiner for individual and population BP [1, 21, 10]. Raised BP (greater than 140/90 mmHg) is the leading cause of mortality and disability in adults worldwide, mainly due to CVD [9]. With increasing BP, the risk for CVD outcomes increases progressively, and starts even at suboptimal BP levels 115/75 mmHg [35;36]. The public health significance is even greater having in mind there are so many individuals in the population with these BP values and the fact that clinical guidelines do not recommend any treatment for the majority of these individuals. It is well established that a reduction in BP causes a significant reduction in vascular events, and therefore, a wide population-approach through non-pharmacological measures (diet and lifestyle) is the most feasible option, recommended by the WHO and adopted under a UN Resolution of the 66th World Health Assembly in 2013 [36;37].

One of the latest epidemiological studies estimates that 9.5% (1.65 million) of all deaths annually from CVD worldwide in 2010 were attributed to sodium intake above the recommended level, 2.0 g of per day. Four of every 5 deaths (84.3%) occurred in low- and middle-income countries, and 2 of every 5 deaths (40.4%) were before 70 years of age [23].

Despite the many advances in our understanding, the precise mechanisms of how dietary salt elevates BP are still poorly understood. Besides mechanisms by which the kidneys retain salt, mechanisms that promote the expansion of extracellular fluid volume and increased cardiac output, many researchers are focused on molecular and biochemical events following endothelial dysfunction and peripheral vascular resistance [39;40]. Excess dietary salt and dietary or endogenous fructose also can play a synergistic role in the development of high sodium-induced hypertension. Several mechanisms promote salt and water retention, and sensitization to the renin-angiotensin system, while promotion of insulin resistance and nitric oxide (NO) deficiency are involved [41].

Gut bacteria could also affect the ability of the kidneys to excrete sodium, contributing to the BP level and control of hypertension. This can be partially explained by the beneficial or non-beneficial effects of short-chain fatty acids (SCFAs) (acetate, butyrate, and propionate/lactate), or by modulation of immunity and inflammation, cell metabolism, and proliferation [42]. High salt intake could additionally drive autoimmunity by inducing T helper (TH)17 cells, which could also contribute to hypertension. Induction of TH17 cells depends on the gut microbiota, yet the effect of salt on the gut microbiome is unknown [43].

### 3.2. Salt and obesity

Obesity has also become a leading public health concern. Recent evidence suggest that, independent of energy intake and high intake of macronutrients (fats, carbohydrates and/or proteins), intake of high non-caloric nutrients, such as micronutrient dietary sodium, could be associated with increased overweight and obesity risk, contributing significantly in metabolic and diabetes risk [44].

Several epidemiological studies have already shown that salt intake, by increasing thirst, promotes passive overconsumption of food and sugar-sweetened beverages [45,46]. A 1 g/d increase in salt intake was associated with an increase in consumption of sugar-sweetened soft drink of 27 g/d in children and adolescents [44,45]. Excessive consumption of processed food that is high in both calories and salt contributes to higher incidences of overweight and obesity [16]. Salty foods are often more palatable, encouraging consumption of greater quantities of these foods.

Dietary fructose intake has been undoubtedly linked to increased *de novo* lipogenesis, when newly synthesized fatty acids are either secreted into the blood as very low density lipoproteins – triglycerides – or temporarily stored as intrahepatic triglycerides, decreased insulin sensitivity and increased visceral adiposity in overweight and obese adults [47].

The newest evidence indicates possible novel pathophysiological mechanisms of obesity, including high fasting ghrelin in healthy individuals consuming a high-salt diet, as well as endogenous fructose



production and leptin resistance in mice. The mechanism for this effect is still unknown. High salt intake led to an increase in osmolality in the liver which triggers the aldose reductase pathway (polyol pathway), resulting in the development of metabolic syndrome, fatty liver and elevated BP in mice. High salt-induced hyperphagia is driven by fructose-dependent hyperleptinemia and reduced hypothalamic leptin sensitivity [48].

High-salt diets have been associated with a higher risk of developing diabetes mellitus regardless of the calorie intake [49,50]. A clinical study performed to assess findings in animal studies confirmed that high baseline salt intake also predicts metabolic syndrome, diabetes and non-alcoholic fatty liver disease in a healthy population, regardless of the calorie intake [48].

#### **4. Salt debate**

For decades, especially since the salt reduction strategy became part of a global strategy to reduce chronic diseases, the efforts to cut dietary salt were met with fierce resistance by the salt industry and some scientific opponents. Low-quality research with conflicting evidence started a debate over the extent to which elevated salt consumption contributes to death. This challenged clinicians and policy stakeholders to keep informed on the effects of salt on health outcomes.

One of the first studies with results opposed to the official recommendations showed a “J-shaped” association between estimated sodium excretion and cardiovascular events in a cohort with 29000 high-risk patients [51]. Mente and associates, in their re-analysis of data from the Prospective Urban Rural Epidemiology (PURE) study, also produced some controversial results [52,53]. Among them, they stated that intake below 3,000 mg of sodium per day was associated with an increased risk of death and that the strong association between salt and stroke exists only in the highest tertile of salt intake (over 12.7 g/day) [52,53]. Meticulous analysis of these results showed serious methodological gaps, such as inaccurate sodium measurements and sample selection (people eating a low sodium diet might be doing so because they are already sick, making it impossible to attribute low sodium intake as a cause of their poor health outcomes) [54].

#### **5. Salt reduction strategies**

Salt reduction has been recognized as the ‘best buy’ approach to prevent and reduce NCD’s [55]. WHO’s global action plan for the prevention and control of NCDs sets a specific target to reduce mean population salt intake of 30% by 2025, with the aim of achieving a target of less than 5 g per day (approximately 2 g sodium) [37].

Modest, stepwise sodium reduction, as recommended by the leading health authorities [1,2,20-21,37,55] remains an achievable, effective, and important public health strategy to prevent a considerable number of heart attacks and strokes and save costs in health care, globally [56]. The latest cost-effectiveness analysis of different national salt reduction policies shows that government-supported national policy to reduce population sodium intake by 10% over 10 years is recognized as cost effective in all countries, globally [56]. New pathophysiological mechanisms implies that reducing salt intake could be important not only for reduction of BP, CVD and stroke but even more, in promoting weight loss and treatment of metabolic consequences.

National salt reduction strategies combine estimation of population salt intake, monitoring of salt content in foods, food reformulation, consumer education, front of pack labeling, interventions in public institution settings, and tax policies for salty foods [20, 58-59]. Food reformulation is one of the key pillars of salt reduction strategies. It is a long and challenging process, having in mind that salt has numerous technological, sensory, behavior and safety impacts [60]. Many technological interventions could contribute to changes in salt concentrations that are acceptable to consumers. A combination of novel technological treatments such as high hydrostatic pressure and ultrasound technology, changing in salt perception, “stealth” reformulation, salt substitutes and enhancers, interaction of senses and the use of flavorings, changes in salt structure, and using different emulsions, seem to be promising to ensure microbiological safety in low-sodium products [60,61].



## 6. Conclusion

There should be no further delay in issuing a national salt reduction strategy. A public health framework given by leading world health authorities, experience and results from numerous neighboring and countries globally, should lead and facilitate progress in Serbia. The Institute of Public Health of Vojvodina, supported by the local and provincial governments, has implemented some activities regarding salt reduction strategies at local and regional levels [62-65]. As a country with high prevalences of obesity and hypertension, and high salt intake, Serbia would greatly benefit from adopting and implementing a national sodium reduction program that minimize risks through education, regulation, and enforcement.

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## Wooden breast – a novel myopathy recognized in broiler chickens

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**Abstract.** Abnormally hard breast fillet consistency began to emerge in commercial broiler chickens around 2010. Due to the remarkable muscle hardness, the condition acquired the vernacular name ‘wooden breast myopathy’. This myopathy starts to develop after two weeks of age at the earliest and typically proceeds into chronic myodegeneration in three to four weeks of age. The lesion begins focally and typically develops into a diffuse lesion that involves the entire major pectoral muscle. The restricted location of wooden breast lesion in the *m. pectoralis major* distinguishes it from several other myodegenerative diseases that widely affect the skeletal muscle system and often the cardiac and smooth muscle systems too. Although industry-wide incidence rates are difficult to assess, it has been estimated that approximately 5-10% of commercially produced breast fillets exhibit severe WB. Even at low incidence rates, the costs to industry are substantial, as breast fillets with the wooden breast condition are often downgraded and sold at a discount, used for further processing, or in extreme cases, discarded. Because the etiology of wooden breast is still unclear, in the future, study of the early lesions, pathogenesis and the possible reduction of animal welfare are likely to gain more attention.

### 1. Introduction

Agricultural production in both crop and animal sectors provides food for people and contributes to food security. Food security is defined as a condition when all people, at any time, can physically and economically access safe and nutritious food that fulfills their needs, is acceptable, enables them to engage in normal life activities, and provides them with a healthy life. Food security depends on many factors, and among the most significant are the increase in the world’s population (seven billion in 2012, eight billion according to projections in 2028), climate change (global warming), environmental pollution, fewer drinking water resources, wars, and population migration. Under such conditions, as we face a population explosion, livestock production should provide sufficient quantities of macronutrients, primarily proteins, and micronutrients (minerals, vitamins). In spite of all the difficulties and while respecting animal welfare, it is considered that production of food of animal origin will continue to increase due to constant improvements in genetic selection, animal diet (feed that can make the most of the genetic potential of animals), health care, farming and care of animals.

In recent years, increasing importance is attached to the poultry industry, primarily broiler chicken production, which has higher annual increases than production of other domestic animals. Nowadays, poultry meat production is equal to pork production worldwide, and there is no doubt that in the years ahead, poultry meat will be the most common type of meat. Today, of the total animal origin protein, the most valuable protein in human nutrition, 45% comes from large ruminants (meat and milk), 31% from poultry (meat and eggs), 20% from pigs, and 4% from small ruminants (milk). The advantage of poultry production is the fact that this animal species has excellent feed conversion efficiency, short generation period (the average is 42 days), and the final product is acceptable in all countries and religions around the world. For one kg of poultry protein, between 18 and 28 kg of dry material is needed, and for one kg of protein derived from ruminants, 133 kg of dry material is needed.

The beginnings of intensive poultry production date back to 100 years ago (the time after World War I), when two lines of chicken hybrids were selected, one of which was directed to eggs (layer strain) and



the other meat (broiler strain). Multi-year selection efforts with heavy hybrid strains focused primarily on increasing the meat yield, especially breast meat with low fat. With modern broiler hybrids, up to 40% of the carcass (hull prepared for barbecue) is breast, and this contains about 1% fat.

Despite all the efforts of producers, conditions related to feed formulation, farming conditions, health care, and biosecurity measures, the safety of poultry meat is not absolute. Today, there are enormous efforts to reduce the frequency of the most common bacterial pathogens in poultry meat (*Salmonella* spp., *Campylobacter* spp., *Staphylococcus* spp.). In Europe, the previously common findings of antibiotics in foods of animal origin are now largely excluded by the prohibition of antibiotics used as growth promoters. Numerous *ante-mortem* and *post-mortem* factors can affect the quality of carcasses (skin changes, joint bleeding, bone fracture, hemorrhages) or meat (PSE and DFD meat, petechiae, wooden breast). In recent years, significant research has been conducted on these meat defects.

## 2. Myopathies in modern poultry

The increase in consumer demand for poultry meat has put pressure on producers to increase production while reducing the cost and duration of production. As a result, broilers are continuously selected to attain greater body weight at younger ages. This is evidenced by the modern broiler chicken gaining on average 20-40 g daily for the first two weeks of life, then approximately 100 g daily until slaughter age (about 42 days), when the broiler is approximately 3 kg liveweight [1,2]. On the other hand, parallel with the development of the body, undesirable traits have emerged such as poor reproduction, ascites syndrome, skeletal abnormalities and impairments of the immune system [3,4,5,6,7,8].

Muscle-related changes are called myopathies and pose a serious problem in the poultry industry. One of these myopathies is “wooden breast” (WB). The name “wooden breast” is related to the characteristic morphological change, i.e., the stiff, hard consistency, observed only on the pectoral musculature (*m. pectoralis major*) [9]. Although histological and morphological analyses indicate there is some similarity of WB to other known myopathies (such as hereditary muscular dystrophy, nutritional and toxic myopathies, deep pectoral myopathy, PSE, and DFD), more detailed studies nevertheless show these are different diseases [10]. WB myopathy most commonly occurs in combination with another previously detected myopathy, the so-called “white striping”, which is the presence of white stripes parallel to the muscle fibers [11,12,13,14,15]. Histologically, it is difficult to distinguish between these two myopathies, but it has been concluded that they are two different diseases, since their individual occurrence is possible [16,17].

The characteristic feature of WB is the hard consistency of the *m. pectoralis major*, without the involvement of other skeletal muscles, smooth muscle, or cardiac muscle, while these other muscle types are involved in other myopathies [18]. The macroscopic hallmarks of WB include the abnormally hardened consistency and pale color of the major pectoral muscle. Additional features include an outbulging appearance of the affected area and sometimes hemorrhage and a layer of clear, slightly gelatinous material covering the muscle. The hardened muscle consistency in WB could be explained by the prominent fibrosis often observed in the chronic phase of WB, although there are findings in which fibrosis has not been confirmed as an independent factor for WB formation. Moreover, some authors suggest myofiber degeneration and swelling together with fibrosis to be responsible for the hardened consistency of WB muscles [18].

## 3. Histological changes in *m. pectoralis major* in WB myopathy

After detecting the characteristic macroscopic changes, confirmed diagnosis of WB requires histological confirmation. The macroscopic WB lesions are strongly associated with polyphasic myodegeneration. The polyphasic lesion type denotes the occurrence of both degenerative and regenerative changes simultaneously within the lesion area. This indicates repeated or progressing damage to the muscle cells and rules out a single pathologic insult as the etiopathogenesis for WB. Histological examination of the lesions in *m. pectoralis major* revealed characteristic features such as an increase in degenerative and atrophic fibers associated with loss of cross striations, variability in fiber size, floccular/vacuolar degeneration and lysis of fibers, mild mineralization, occasional regeneration (nuclear rowing and multinucleated cells), mononuclear cell infiltration, lipidosis, interstitial inflammation, and fibrosis [9,12]. WB starts to develop after two weeks of age at the earliest and typically proceeds into a chronic myodegeneration at three to four weeks of age, but muscle degeneration is most pronounced from 5-6

weeks of age (slaughter period). The early macroscopic lesions are usually focal and exhibit mild muscle firmness compared to the more severe and typically diffuse lesions in the older birds. A gradual decrease in the histopathological lesions was detected moving from the surface towards the deep portion of the muscle, with the first exhibiting profound degenerative myopathic lesions accompanied by the replacement of chronically damaged muscle with adipocytes and fibrosis [19].

#### 4. Etiology of the formation of wooden breasts and consequences

Although numerous studies have speculated about the etiology of this myopathy, it is still unclear. Some of the possible factors that could initiate the myopathic changes are reduced ability to store/utilize carbohydrate as an energy source [20,21,22], accumulation of calcium ions [23,24], hypoxia and oxidative stress [25], and circulatory insufficiency in these fast-growing birds [26]. A single factor that has been consistently associated with the incidence of these myopathies is heavier body weight and thicker fillets in broilers [27,28,29].

One of the most important factors for the development of WB myopathy in rapidly growing broiler chickens is metabolic distress resulting from hypertrophic muscle fibers that increases the diffusion distance between blood vessels and muscle fibers [30]. It has also been hypothesized that in poultry selected for meat production, the growth of the connective tissue in muscle does not keep pace with muscle fiber radial growth, and the fibers outgrow the supporting connective tissue, leading to muscle damage [31,32].

The degree of myodegeneration also affects changes in the muscle's chemical components, and the ability to process the meat. Meat quality is the direct result of muscle morphologic structure and cellular biologic processes regulating muscle development and growth. The poultry industry has made substantial genetic improvements in growth rate and breast meat yield. However, these increases have changed both the morphometry and cell biology of the *m. pectoralis major*. In general, growth selection has resulted in increased degeneration of muscle fibers [33], larger diameter muscle fibers (fast-growing male meat chickens have *m. pectoralis major* muscle fibers three to five times wider than slower growing birds) [34], decreased capillary blood supply to the muscle [35], reduced connective tissue spacing between muscle fiber bundles and muscle fibers [34,35], and increased myofiber degeneration [36].

WB occurs most frequently in fast-growing, high breast-yielding broiler strains. The incidence of WB also seems to be higher in broilers that are male, on high nutrient diets, or slaughtered at older ages and heavier weights [28,35,37,38]. Gait score is a good predictor for broiler mobility [39], and walking ability is usually weaker in heavier [40,41] and faster growing broilers [41]. In some studies, reduced wing mobility was linked to the myodegenerative WB lesions [42,43]. This arouses the suspicion that pain or discomfort occurs as a result of heightened sensitivity of the breast area in WB. Although the amount of pain associated with WB is difficult to demonstrate from the various studies, severe myodegenerative lesions in humans have been reported to cause substantial pain [44].

#### 5. The influence of WB on the quality of broiler meat

WB, together with other myopathies (WS, PSE, vitamin E and Se deficiency, etc.) reflects primarily on meat quality [27]. Although these quality issues do not impose microbiological or other food safety risks, they render the products less attractive to the consumer [12]. The duration of broilers' lives affects the concentration of chemical components in the breast and leg muscles, resulting in more attractive sensory flavor properties in longer-lived birds.

*M. pectoralis major* is the most valuable part of the broiler carcass and constitutes approximately one fifth of the total body weight [45,46]. Structural changes in WB fillets have an overall negative effect on the meat quality. Consumers perceive increased fat deposits in breast fillet as unfavorable, as they give the impression the meat is "unhealthy" [12]. Moreover, increasing fat (which is hydrophobic) can further reduce the ability of the flesh to bind water. WB is known to impair the water-holding capacity of the breast meat. Fresh WB fillets exhibit more drip loss during refrigerated storage and greater cooking loss [47]. The pale color, soft consistency and poor water-holding capacity are caused by the denaturation of myofibrillar proteins due to rapid *post-mortem* decline of pH [48] when meat is still warm, before the temperature lowers during processing.

Texture analyses show fresh WB fillets have a high compression force. After thermal cooking treatment, WB fillets are harder, more rubbery, more elastic, and have greater resistance to chewing than

normal fillets. Generally, the change in the texture profile is a feature of complex chemical changes in the muscle fibers and connective tissue that accompany WB [49].

## 6. Conclusion

WB myopathy is a newly discovered meat quality defect in fast-growing meat chickens that is only observed *post-mortem*. One of the major challenges in research on WB is the lack of an effective biomarker to identify the condition in live birds. Although there was an attempt to assess the presence of WB by palatalizing the pectoral musculature, this was not effective. The second challenge is the lack of an effective, standardized scoring scale, as the WB scoring systems currently used are subjective. To date, the majority of published studies on WB have focused on the chronic and *post-mortem* changes, and the meat quality issues. In the future, study of the early lesions, pathogenesis and the possible reduction of animal welfare associated with WB are likely to gain more attention.

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# Preservation of meat and meat products using nanoencapsulated thyme and oregano essential oils

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**Abstract.** Among other plants, thyme and oregano are commonly used in Mediterranean cuisine, especially in meat dishes. Although the essential oils of these two plants possess great antimicrobial and antioxidative properties, their application as natural meat preservatives are limited due to hydrophobicity, sensitivity to external factors and interaction with food components. Furthermore, essential oils can have adverse impacts on meat's organoleptic properties. A possible way to overcome these barriers is by incorporating essential oils into nanometric delivery systems. Nano-sizing essential oils increases their stability, protects them, and allows their controlled release. This enhances the bioavailability of the essential oils and reduces their possible adverse impact on meat products' organoleptic properties by preventing their unwanted interactions with food components. The antibacterial and antioxidative effect of nanoencapsulated essential oils is confirmed in numerous studies, and some of them show that in this form, essential oils were potent in food models e.g. beef burgers, pâté and rainbow trout. However, a more promising way to introduce nano forms of essential oils into foods is incorporating them in packaging systems.

## 1. Introduction

Microbial contamination and lipid oxidation are the main causes of meat deterioration. Moreover, meat and meat products are common sources of foodborne pathogens and the consumption of contaminated products are frequently linked to *Salmonella*, *Campylobacter*, *Listeria*, *Escherichia coli* and *Staphylococcus aureus* outbreaks [1, 2]. Another safety issue for meat production is the use of synthetic additives, which can have potential toxic and negative side effects [3]. Thus, the meat industry has to meet safety, hygienic and quality criteria as well as consumers demand for healthy, natural and minimally processed food.

Among other herbs, oregano and thyme belonging to the *Lamiaceae* family are the most commonly consumed, almost essential components of Mediterranean cuisine [4, 5]. Essential oils (EOs) obtained from these plants are of great interest in the food industry as in the other fields, due to their significant antimicrobial and antioxidative activities [6]. Moreover, these EOs and their main constituents, the phenolic compounds carvacrol and thymol, do not have a mutagenic effect on human lymphocytes [6, 7], and are categorized as generally recognized as safe (GRAS), which makes them suitable candidates for food preservation.

However, application of EOs in their free (original) form is limited because of their hydrophobicity, which prevents them from dissolving in food's aqueous phase, and their volatility, causing losses during food processing and consequently leading to increased cost of production processes [8]. Interaction with meat components can reduce or completely inhibit the activity of EOs



[9]. Phenolic compounds can attach to food proteins, decreasing the amount of phenolics available for bacterial growth inhibition [10]. EOs are affected by numerous factors including pH, water activity, enzymes, temperature, relative humidity, and storage conditions [11]. Furthermore, some EOs have negative effects on sensory properties of food products, depending on the main compounds of the EO, concentration, and the type of food [12].

Nanoencapsulating EOs is one way to overcome these barriers and allow EOs to exhibit their full preservative potential in food [11]. Nanoencapsulation protects the EOs against unfavorable conditions during processing, storage, and transport [13, 14]. The advantages of nanoencapsulated EOs are their stability and controlled release, which enhances their bioavailability and reduces possible adverse impact on meat products' organoleptic properties [11, 15].

## 2. Nanoencapsulation

Nanoencapsulation is a new technology and refers to coating the active agent (in this case, EO) within another material at sizes on the nano scale [16]. Materials for nanoencapsulation need to meet a wide range of requirements to be approved by the food sector [17]. All coating materials used for this purpose should be categorized as GRAS [18], should protect the EO and preserve its activity, be nonreactive with the EO or with food components and be biodegradable. The coating materials are mainly polysaccharides of plant origin such as starch and cellulose and their derivatives, different types of gums, galactomannans, pectins, soluble soybean polysaccharides, and polysaccharides of microbial and animal origin including dextran, chitosan, xanthan, and gellan. Moreover, some lipids (fatty acids, fatty alcohols, waxes, glycerides, and phospholipids) and proteins (whey proteins, caseins, gelatin, gluten, zein, albumin, globulin, and silk fibroin) can be used for this purpose [11, 17, 19].

In general, nanoencapsulation techniques use two main approaches: top-down and bottom-up [20]. Top-down approaches induce particle size decreases during the encapsulation process and are high-energy, while bottom-up approaches are low-energy and refer to construction of nano-sized materials *via* self-assembly and self-organization [20, 21]. Although only some nanoencapsulation methods can be used to encapsulate hydrophilic substances, all methods can be used for encapsulating lipophilic compounds (i.e. EOs) [17, 22].

The techniques used for nanoencapsulating EOs can be also classified as producing lipid-based nanoparticles or polymer-based nanoparticles. EOs are lipophilic compounds and, therefore, lipid-based systems mostly produce nanoscale EOs for food preservation purposes [11]. Lipid-based nanoparticles are nanoemulsions, nanoliposomes, solid lipid nanoparticles (SLN), or nanostructured lipid carriers (NLC) [17].

Nanoemulsions are liquid-in-liquid dispersions with sizes in the order of 100 nm [23]. There are two possible means of nanoemulsion preparation: top-down approaches which include high pressure homogenization, micro fluidization, or ultrasonication and bottom-up approaches such as phase inversion and spontaneous emulsification methods [20].

Nanoliposomes, spherical lipid vesicles with an aqueous core and amphiphilic lipid bilayer [24], are another form of nanoscale delivery system for EOs. The advantage of nanoliposomes is that they easily fuse with bacterial membranes and enter the bacterial cell [20], while their main disadvantage is their stability. However, coating nanoliposomes improves their stability, thereby prolonging their half-life [20, 25].

Hot homogenization and cold homogenization are techniques used to prepared SLNs and NLCs [17]. As the name suggests, SLNs are made from solid lipids such as fatty acids, triglycerides, steroids, partial glycerides, and waxes [24].

Polymeric nanoparticles are classified as nanocapsules and nanospheres. Nanocapsules consist of a polymeric shell, a core(s) and active agents, in this case EOs, which can be placed inside the core or adsorbed on the surface, producing a reservoir-type nanomaterial. Nanospheres are matrix-type systems in which the EO is homogeneously dispersed in the structure [17]. Moreover, reservoir-type and matrix-type encapsulations are classified according to the mechanism by which the active agent is released. While the reservoir-type has a capsule surrounding the active agent, which is placed in one or

multiple cores, in the matrix-type, the active agent is dispersed in the polymer phase or carrier material and it is also present at the surface. Release of the encapsulated active agent from the reservoir-type is due to pressure, whereas in the matrix-type, the active agent is released *via* diffusion of active agents or erosion of the matrix. In both types of nanomaterial, release is influenced by solubility, diffusion, biodegradation of the shell and matrix materials and the size of encapsulate [17, 26, 27].

### 3. Antibacterial mechanisms of free form and nanoencapsulated EOs

The antibacterial effect of free form EOs is attributed to several mechanisms. EOs interact with lipids in microbial cell and mitochondrial membranes, increase cell permeability, change membrane potential, cause ion loss and collapse of the proton pump, and disturb microbial metabolism leading to lysis and microbial death [12, 28, 29]. However, the exact mode of action of nanoencapsulated EOs is still not completely elucidated. It is supposed that nanoencapsulation enhances EO activity due to the reduced size, allowing nano-EOs to interact more efficiently with cell membranes [8] by increasing the surface area per unit of mass [29]. Consequently, lower doses of EOs can be used [30]. Apart from the active agent, some carriers used in nanomaterial production also possess antimicrobial activity, change membrane potential, generate reactive oxygen species and affect microbial metabolism [31]. The antimicrobial activity of thyme EO (TEO) and oregano EO (OEO) is attributed to their main phenolic components, thymol and carvacrol, but it is supposed that a synergistic effect is achieved with minor components, including the monoterpene hydrocarbons *p*-cymene and  $\gamma$ -terpinene [6]. Furthermore, some authors suggest that the nano-EOs could act synergistically with the carrier used for nanoencapsulation. Materials used for encapsulation protect EOs from reacting with the food matrix and transfer nano-EOs to specific targeted sites like water-rich phases [11].

### 4. Practical application

Numerous studies reported *in vitro* antibacterial and antioxidative effect of nanoencapsulated TEO and OEO and their predominant components, thymol and carvacrol.

Sotelo-Boyás *et al.* [32] reported that nano-TEO, composed mainly of thymol and carvacrol encapsulated in chitosan and with an average size of 9.1 nm, exhibited activity against *S. aureus*, *L. monocytogenes*, *Bacillus cereus*, *Salmonella* Typhi, *Shigella dysenteriae* and *E. coli*. However, the highest inhibitory activity was observed against *B. cereus* (inhibition halo 1.9 cm) for 40  $\mu$ L of MIV. Furthermore, Moghimi *et al.* [33] investigated the antibacterial effect of *Thymus daenensis* EO in both free and nanoemulsion forms by measuring the minimum inhibitory concentration (MIC) and minimum bactericidal concentration. The antibacterial activity of the TEO against *E. coli* was significantly greater when it was converted into a nanoemulsion.

However, there are very few data available in the literature about the effect of TEO and OEO and/or their nanoforms in the meat and meat products. Thyme EO-loaded chitosan nanoparticles were prepared by a two-step process including oil/water emulsion and ionic gelation and their effect on microbiological, chemical quality and organoleptic properties of beef burgers during 8 days of storage was studied [34]. Nanoencapsulated TEO added at concentrations of 0.1% and 0.05% significantly inhibited microbial growth. The burgers with added nanoencapsulated TEO had lower counts of *Enterobacteriaceae*, *S. aureus*, total mesophilic viable counts, and yeasts and molds than control burgers and burgers with free form TEO. Addition of nanoencapsulated TEO resulted in lower concentrations of thiobarbituric acid reactive substances (TBARS) in the burgers. The burgers containing nanoencapsulated TEO had overall acceptability scores similar to those containing free form TEO and control burgers. Based on the results of this study, encapsulation of TEO in chitosan nanoparticles could potentially control microbial and chemical alterations in the burgers and did not have the strong odor of free form EO, thus improving the sensory quality of the meat products [34].

Similarly, Moraes-Lovison *et al.* [35] reported that nanoemulsions encapsulating OEO could be incorporated into pâtés to prolong their shelf life. MIC and minimum bactericidal concentration (MBC) of OEO nanoemulsions with average droplet diameters of 35 to 55 nm were assessed against *S.*

*aureus* and *E. coli*. MIC and MBC did not differ significantly during 90 days of storage under refrigeration conditions, indicating the long storage period did not affect stability and antibacterial properties of the nanoemulsions. When added into chicken pâté and stored for 45 days, numbers of *E. coli* and *S. aureus* were reduced. While the antibacterial effect of nano-OEO against *E. coli* was greater than reported for free form OEO, the effect against *S. aureus* was the same for both nanoencapsulated and free form OEO. The incorporation of nanoemulsions in chicken pâté did not change the physicochemical characteristics of the meat product [35].

Moreover, EO nanoemulsions can be used as a preservative for fish meat. Ozogul *et al.* [36] examined the effects of various EO nanoemulsions on microbiological and chemical quality and sensory attributes of rainbow trout (*Oncorhynchus mykiss*) fillets during 24 days storage at 2 °C. TBARS levels were higher in the control than in the fish with nano-TEO, indicating TEO nanoemulsion protected the fish against oxidation. Among the EO nanoemulsions examined, rosemary and thyme nano-EOs slowed the growth of mesophilic bacteria, psychotropic bacteria and *Enterobacteriaceae*. Nano-TEO's main components were carvacrol (71.54%) and *p*-cymene (11.84%), and the nanomaterial extended the shelf life of rainbow trout by approximately 3 days.

Incorporating nano-EOs in packaging systems is a more promising way to introduce nanoencapsulated EOs and their active compounds into food [37]. Moreover, edible films containing active agents such as EOs could be considered as an alternative form of food packaging [30]. Edible films obtained from alginate-based nanoemulsions loaded with TEO exhibited strong antibacterial effects against *E. coli* and reduced the count of these bacteria by 4.71 logs within 12 h [30].

Good hygiene of food contact surfaces in the food chain can help prevent the occurrence of foodborne outbreaks of disease. Microbial adhesion to food contact surfaces and biofilm formation are the main sources of cross-contamination of food [38, 39]. Engel *et al.* [40] examined the effect of 1 and 10 min contact times of liposome-encapsulated thymol and carvacrol with average particle diameter of 270 nm against cocktails of four different strains of *Salmonella* and *S. aureus* adhered to stainless steel. Free form thymol or carvacrol reduced *S. aureus* and *Salmonella* to levels below the limit of detection after 1 min. Thymol and carvacrol nanoliposomes produced similar effects to the free form compounds after 10 minutes. The longer time needed for thymol and carvacrol nanoliposomes to produce antibacterial effects reflects the gradual release of these active agents from the nanoliposomes.

## 5. Precautionary and research points regarding nano-EOs

Using nano-EOs as preservative agents in meats has numerous advantages over using free form EOs, especially the enhanced antimicrobial activity of nano-EOs and their limited effects on the sensory properties of meat. However, there is a lack of data on the effects of nanomaterials on mechanisms of absorption, metabolism, and human health, while the environmental impact of nano-sized particles remains an issue of great concern. Thus, further studies on the safety of nanomaterials are needed before incorporating nanoencapsulated EOs as natural preservatives in food.

## Acknowledgment

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## Household food waste in Belgrade - sin and unconcern

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**Abstract.** The aim of this study was to examine the actual procedures with food in households and consumer attitudes about food waste. The survey was conducted in 83 households in Belgrade, Serbia. All participants were interviewed using a standardized questionnaire. The results obtained show that awareness of food waste is at a satisfactory level, but the actual situation is that food is discarded in large quantities, even though people are aware of what a global problem this is. Large contradictions were observed among the respondents' answers in this study. Respondents who stated that they never discard food, in further responses, declared they throw away significant amounts of food for various reasons (too long storage, overconsumption, improper preparation, etc.). We conclude that people are either unaware of how much food they discard, or they do not want to admit it to themselves. However, participants largely have a sense of guilt about discarding food. This indicates consumer awareness of food waste, and is an encouraging sign that further education could be effective in consumers taking into account their food waste habits, starting from procurement planning, through storage and preparation.

### 1. Introduction

Food is precious and many resources are needed for its production. According to current estimates, globally, about one-third of food produced for human consumption is unnecessarily thrown away or lost, which brings with it an economic cost and cost associated with the environment [1,2]. Unnecessary food waste is a global problem that has grown in recent years and is now being raised in public and political programs. Its importance will continue to grow, especially taking into account the need to feed the ever-increasing population of the world [3,4].

Due to the increasing number of published papers on the topic of food waste in households [5], consumer awareness should be increased as well as their role in this global problem. Are consumers



truly aware of the problems caused by the large amounts of household food thrown away, and are they willing to do something in order to reduce this amount? Surveys are not adequate tools for measuring the amount of wasted food [6]. Due to growing interest in the world, and in Serbia, food waste has become a very popular topic in the media, but also in organizations that want to draw attention to the seriousness of this situation. Is the level of discarded food similar in all parts of the world, whether in emerging countries or not, and are consumers becoming more aware of food waste? The aim of this study was to examine respondents' attitudes about food waste, habits and procedures with surplus food in households and to consider how to increase consumer awareness of the actual quantities of food wasted.

## **2. Materials and Methods**

### *2.1. Investigation and Data Collection*

The study was conducted during a six-month period, from November to April 2018/2019, and included 83 households in Belgrade, Serbia. All respondents were over 18 years old. Households were chosen randomly, with the consent of the main person responsible for the purchase and preparation of food. Participants were briefly informed about the aim of this survey, and the answers and collected data were anonymous.

Experienced researchers prepared the questionnaire and survey structure. In total, the sample size for this survey was 100 households. Since 17 persons did not complete the questionnaire, the final sample consists of 83 households that answered the questions asked. The results obtained are based on self-reporting, and they providing a picture about respondents' habits in shopping, storage behaviours, their attitudes about food waste and actual procedures with surplus food.

### *2.2. Statistical analysis*

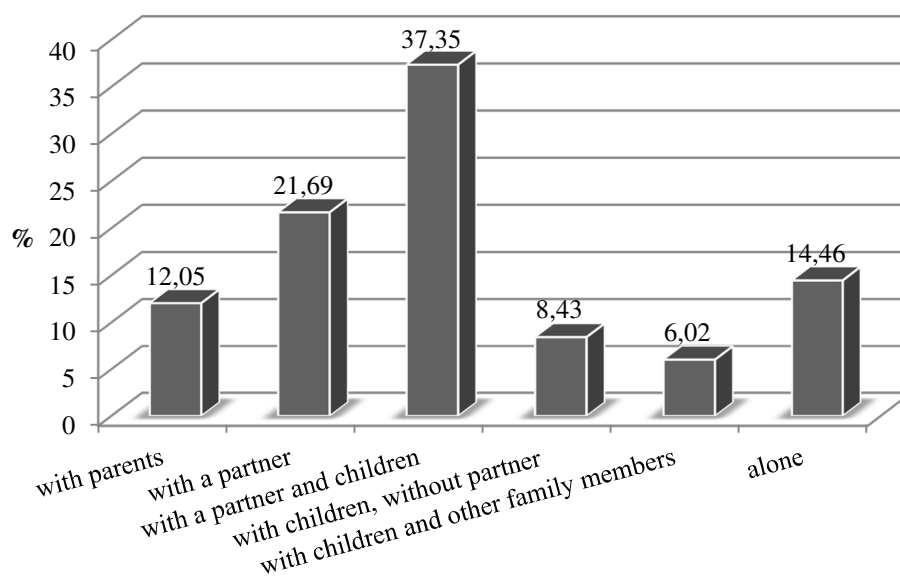
Statistical analysis of the results was elaborated using IBM software, SPSS Statistics 24. Statistical analysis was performed using the Chi squared test to determine the significance of differences between means. Correlations between parameters were used to determined significance of their relationships. A level of 0.05 was considered significant.

To compare food waste in households, the sum of ranks were calculated. First, the data in compared groups were initially arranged and listed in order of increasing value, where the number 1 represents the food that is most often thrown away. Then, sums of ranks for some foods were compared [7].

## **3. Results and Discussion**

### *3.1. Household demographics*

Of 83 respondents, 25.30% were men, and 74.70% women, which is understandable, since women are mostly responsible for household chores in Belgrade. The age groups of respondents (26-35, 36-45, 46-55, 56-65 years) were evenly distributed (20.48%, 22.89%, 20.48%, 21.69%, respectively), with the exception of the youngest (18-25 years, 10.84%) and the oldest (>65 years, 3.61%). The majority of respondents were divided into two levels of education: faculty level (33.73%) and secondary school (36.14%). The majority of respondents (74.70%) were employed full-time. The numbers of household members varied, although most households contained between 2 and 4 members (31.33%, 21.69%, and 20.48%, respectively). The structure of the surveyed households is presented in Figure 1.



**Figure 1.** Household structure in the examined households

### 3.2. Food supply habits

Altogether, 75.90% of the participants claimed they buy food in super/hypermarket, which was significantly more ( $P > 0.05$ ) than those who buy food at a minimarket or local store (16.87%) or at a green market (7.23%). Few participants shopped once or twice a month. Food supply habits are shown in Table 1.

**Table 1.** Food supply habits in households

Question	Answer (%)
How often do you go to a retail outlet to purchase food (three or more products)?	Every day
	27.71
	Every other day
	22.89
	Three times a week
	20.48
How much money per month do you spend on food?	Once a week
	26.51
	Twice a month
	0.00
	Once a month
	2.41
Do you prepare a list of those food items you intend to purchase?	Up to 10,000 RSD
	13.25
	10,000-20,000 RSD
	21.69
	20,000-30,000 RSD
Are you attracted to special offers (promotions, discounts) of foods that you did not plan to buy?	24.10
	30,000-40,000 RSD
	30.12
Do you pay attention to the expiration date on the label?	More than 40,000 RSD
	10.84
Do you buy food that has passed its expiration date (at a discount)?	Yes
	50.60
	No
	49.40
	Yes
	73.49
	No
	26.51
	Yes
	80.72
	No
	19.28
	Yes
	36.14
	No
	63.86

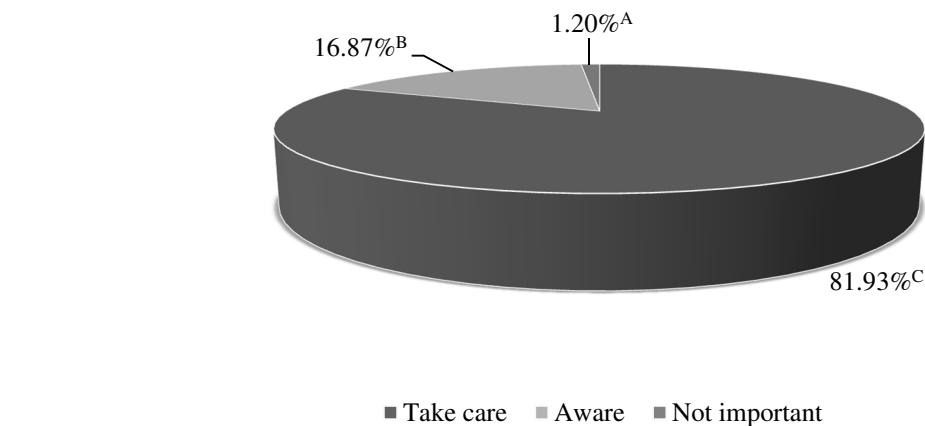
In Table 2, procedures with surplus food in households are given. Reported procedures with surplus foods indicate consumers care about the amount of food wasted, but survey questions did not previously prove to be a valid method for determining people's general food waste habits [8,4,6,9]. Many researchers suggest that the amount of wasted food can best be calculated by combining different methods [4,10,11].

**Table 2.** Procedures with surplus food in households

Question	Answer (%)
Which foods do you discard more often (in greater quantities on a weekly basis)	Cooked food (prepared in household)
	63.86
	Uncooked food
	36.14
What do you usually do with surplus food?	Discard into a container
	14.46
	Leave in a prominent place (next to a container, etc.)
	22.89
How often do you waste food?	Give to a pet (dog, cat)
	31.33
	Feed animals (wild birds, animals kept for breeding, etc.)
	6.02
When preparing larger amounts of food than usual for your household, do you discard large amounts of food?	Rarely have food surpluses
	25.30
	Never
	19.28
What is the most common reason for discarded food in your household?	Once a week
	56.63
	Twice a week
	15.66
What is the most common reason for the expiry date causing you to discard food?	More than twice a week
	8.43
	Yes
	32.53
What is the most common reason for the expiry date causing you to discard food?	No
	67.47
	Expiry date has passed
	18.07
What is the most common reason for the expiry date causing you to discard food?	Altered smell and taste typical of the food
	55.42
	The appearance of the mould
	16.87
What is the most common reason for the expiry date causing you to discard food?	Poorly prepared food
	3.61
	Incorrectly stored food
	6.02
What is the most common reason for the expiry date causing you to discard food?	Expiry date on the food is unclear
	14.46
	Food was stored too long
	30.12
What is the most common reason for the expiry date causing you to discard food?	Food was purchased/cooked in excessive amounts
	34.94
	Food packaging size is too large
	20.48

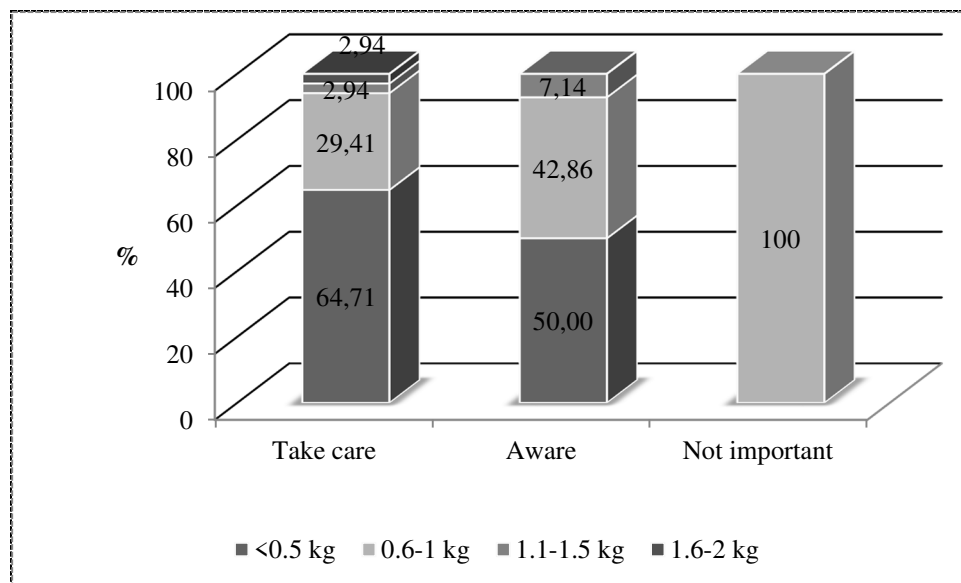
There were large discrepancies between reported and actual practice procedures. Participant awareness of the food waste problem is one thing, but their actions are quite another (Figures 2 and 3). As we can see, significantly ( $P < 0.05$ ) fewer respondents believe that food waste is unimportant (1.20%) than those who are aware of the problems caused by food waste, but will not change their attitude

towards food surpluses in household (16.87%) and respondents who claim to care about food surpluses and avoid food waste (81.93%). However, the same groups of respondents reported they discard significant amounts of food, regardless of their reports about their attitude towards food waste (Figure 3). Porpino et al. [12] also noted that consumers believe food waste is an improper behaviour and generally claim to not waste a lot of food [13]. Most researchers agree that consumers' concerns are the main prerequisite for food waste reduction in households [14-17].



Legend: Different letter <sup>A,B,C</sup>- $P < 0.05$ .

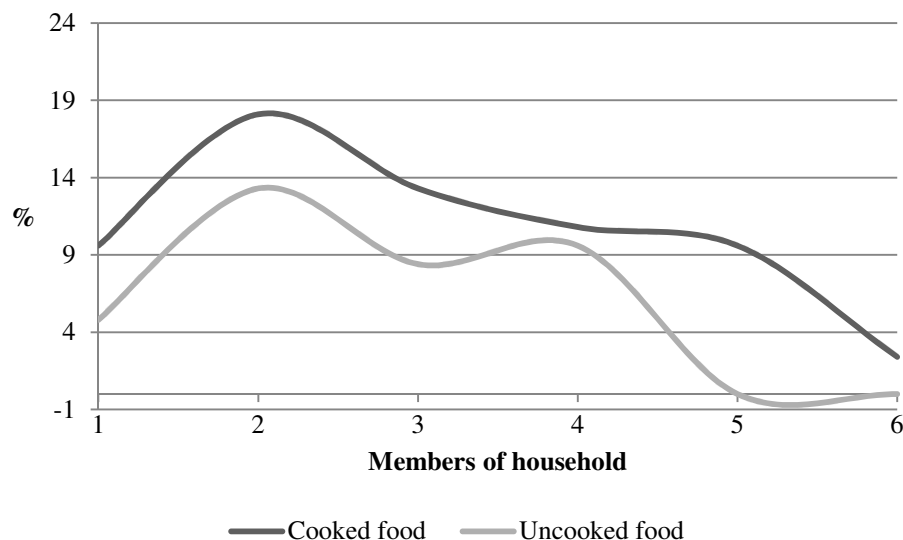
**Figure 2.** Consumer awareness of food waste in households



**Figure 3.** Reported quantity of food wasted weekly in accordance with participants' attitudes towards food waste

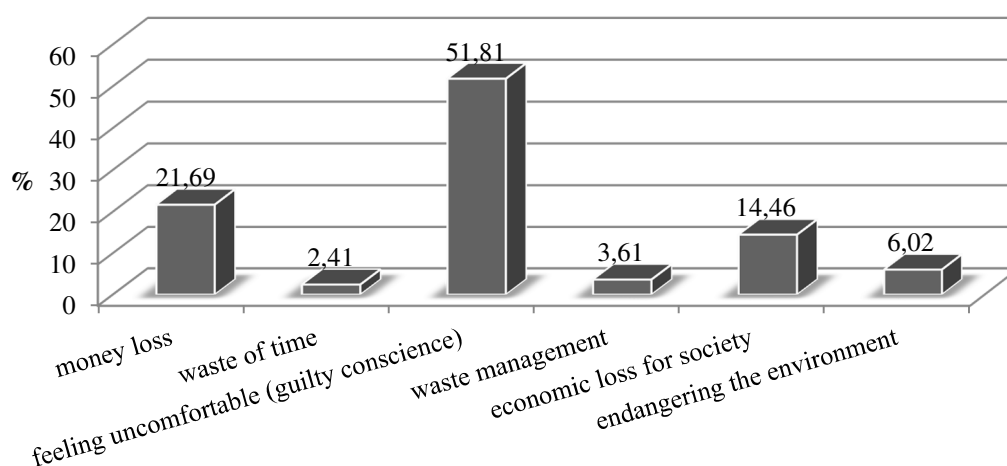
The relationship between the number of household members and the type of food wasted is shown in Figure 4. There was no significant correlation between these two parameters ( $P=0.216$ ), but the data reveal peak quantities of cooked and uncooked food waste in households with two members. We can only speculate on the reason for this, and perhaps this household size is linked to family structure (age and sex), which could affect food waste. In households with more members (5 and more), we speculate the variety of food preferences also increases, and therefore, less food is wasted because leftovers are

more likely to be consumed (someone always likes something). Other researchers explained the discarding of larger quantities of cooked food [18,19] as due to overly large quantities being prepared or served.



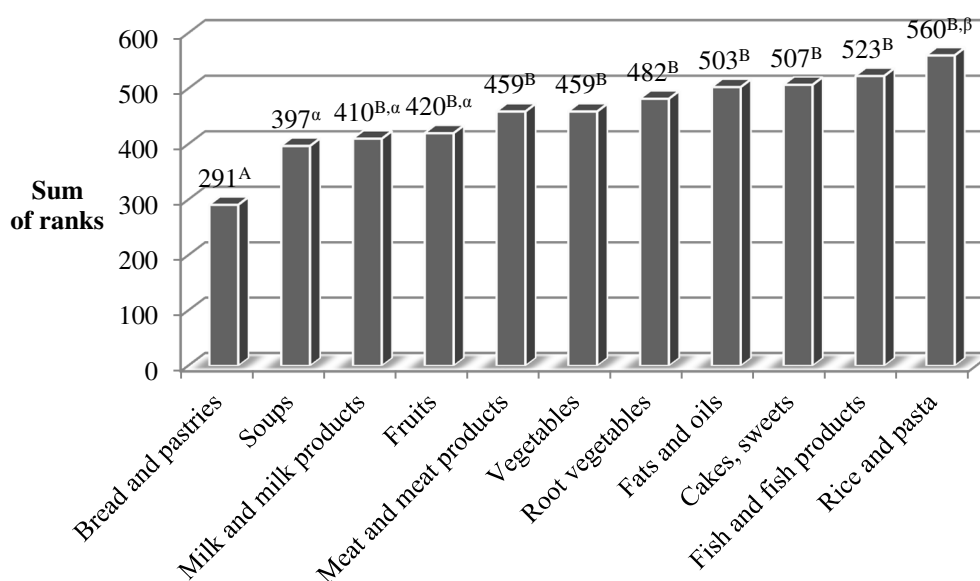
**Figure 4.** The relationship between the number of household members and quantity of food wasted

There were no significant relationships between the examined parameters, which could indicate participant dishonesty in responding, or respondents really being unaware of how much food they wasted. Further processing of the data obtained showed there were differences between the individual tested parameters, but the relationships between them were not determined because of the non-conformity of the response (for example, those who declared they did not discard food at all replied later in the survey that they discarded more than 0.5 kg of food per week). We can conclude that participants were unaware of the amount of food they waste, and that most of the respondents regard food waste as a sin (Figure 5). The reasons participants would consider reducing their food waste in households are given in Figure 5. This result is contradicted by one study [20], but that study produced quite different results from other studies [21-23]. However, there are similarities in other surveys to ours (Figure 5), where endangering the environment ranked behind other food waste problems [20,21,24]. The results obtained show the need for education about the negative environmental impact of wasted food.



**Figure 5.** The reasons participants would consider reducing food waste in households

The respondents reported how much and which foods they threw away. As we can see in Figure 6, significantly more ( $P<0.05$ ) bread is thrown away than other foods (except soups), and significantly more ( $P<0.05$ ) soups, milk and milk products, and fruits are wasted than rice and pasta. Results of another study [19] do not coincide with our research, as respondents in that study declared they mostly discarded vegetables, dairy products, and then bread, milk, and meat. Aschemann-Witzel et al. [25] listed the frequency of categories of wasted food, and their results are also quite different to ours. Some (10.4%) respondents in [25] reported they did not discard food at all, whereas in our study, 8.43% of participants stated they did not discard food.



Legend: Different letter A, B or α, β –  $P<0.05$ .

**Figure 6.** Sum of ranks for food types wasted by Belgrade households (n=76)

#### 4. Conclusion

This pilot study was to obtain information on how suitable this method of survey is, and whether the results relate to the actual state of food waste or to the opinion of the respondents about their own food waste. Further studies will include a larger number of participants and modify the questionnaire according to our experiences. We determined that food waste is largely considered a sin, and believe this could contribute to the real amount of food wasted not being reported. There is insufficient awareness among participants of the amount of food they throw away and the consequences this causes. In any case, it is necessary to increase the level of consumer awareness about the importance of reducing food waste in households.

#### Acknowledgment

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# Managing allergies in food service

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**Abstract.** Food allergens have appeared in the last two decades as a concealed form of threat which significantly endangers public health, and so allergen labelling on food products, drinks and non pre-packed gastronomic products is clearly defined by legal regulations. Appropriately managing food allergies has become an issue for the foodservice industry because of the rising number of individuals with food allergies. Establishing proper communication between and among customers and foodservice employees could be one of the most important steps in preventing food allergy reactions in restaurants. Proper risk communication often initiates increased attention among restaurant staff to ensure customer safety. Current initiatives to support consumers at risk include a recognised standard for manufacturers seeking to eliminate an allergen from their production, and the integration of food allergy into training for caterers and food standards enforcement professionals.

## 1. Introduction

Food allergy is defined as an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food [1] or as an adverse reaction to food in which immunologic mechanisms have been demonstrated [2]. Food allergy is a relatively recent newcomer to the ranks of food safety issues, only being effectively recognised as such in the last 25 to 30 years. This recognition, allied with the near impossibility of avoiding the unintended presence of small, yet potentially dangerous residues of allergenic constituents, brought with it the need to assess and manage the resulting risk. Food allergy is recognised as an important public health issue, requiring collaboration between multiple stakeholders, including the food industry, to be effectively addressed. Food allergens affect the health and life of people with hypersensitivity caused by some food components, and such allergens are identified as severe food safety hazards, so their management is one of the fundamental areas of food safety management systems [3,4,5]. Management of allergens in food production aims to assure the safety of allergic consumers, but has proved particularly challenging because of the unique attributes of food allergy and food allergens. Accurate allergen labelling, minimising unintended allergen presence, and circumspect use of meaningful precautionary labelling where required form the cornerstones of effective allergen management. Allergen control plans are the practical description of the essential measures needed to assure implementation of suitable controls. Food allergen risk management must also be integrated into general food safety management and allergen risks be considered at all stages of the food manufacturing process.

Advantages of providing correct food and beverage service to consumers with food allergies include increasing sales, respect and loyalty of consumers [6]. According to [7], research worldwide



indicates a lack of knowledge by hospitality employees about the risks of allergenic food ingredients. The worldwide research also indicates the fact that approximately 70% of hospitality employees have not had an opportunity to be trained and educated about food allergens, allergenic food ingredients or allergen management [8,9].

According to the Serbian *Rulebook on Labeling, Marking and Advertising of Food* [10], in food service, during the presentation of the food offered to the final consumer and before eating, hospitality employees should highlight all necessary information about the presence of ingredients that can cause allergies and/or intolerances determined in accordance with these regulations.

## 2. The 14 allergens

The 14 allergens as listed according to [11] are:

1. Cereals containing gluten, specifically wheat (including spelt and Khorasan wheat), rye, barley, oats and their hybridised strains and products thereof, except: a) wheat based glucose syrups including dextrose b) wheat-based maltodextrins c) glucose syrups based on barley d) cereals used for making alcoholic distillates including ethyl alcohol of agricultural origin;
2. Crustaceans and products thereof (for example prawns, lobster, crabs and crayfish);
3. Egg and products thereof;
4. Fish and products thereof, except: a) fish gelatine used as carrier for vitamin or carotenoid preparations b) fish gelatine or isinglass used as a fining agent in beer and wine;
5. Peanuts and products thereof;
6. Soybeans and products thereof, except: a) fully refined soybean oil and fat b) natural mixed tocopherols (E306), natural D-alpha tocopherols, natural D-alpha tocopherol acetate and natural D-alpha tocopherol succinate from soybean sources c) vegetable oils derived phytosterols and phytosterol esters from soybean sources d) plant stanol esters produced from vegetable oil sterols from soybean sources;
7. Milk and products thereof (including lactose), except: a) whey used for making alcoholic distillates including ethyl alcohol of agricultural origin b) lactitol;
8. Nuts (specifically: almonds (*Amygdalus communis*), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoensis* (Wangenh.) K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia or Queensland nuts (*Macadamia ternifolia*), and products thereof, except for nuts used for making alcoholic distillates including ethyl alcohol of agricultural origin;
9. Celery and products thereof;
10. Mustard and products thereof;
11. Sesame seeds and products thereof;
12. Sulphur dioxide and/or sulphites at concentrations of more than 10 mg/kg or 10 mg/L in terms of the total SO<sub>2</sub> which are to be calculated for products as proposed ready for consumption or as reconstituted according to the instructions of the manufacturers;
13. Lupin and products thereof, and;
14. Molluscs and products thereof (for example mussels, clams, oysters, scallops, snails and squid).

## 3. Food allergens and sensitivities

Food allergies and sensitivities are illnesses that affect individuals in the population when eating foods or food ingredients that most consumers can tolerate with no problem [12]. These illnesses are sometimes called individualistic adverse reactions to foods because they affect only certain individuals in the population. Another catch-all term for these individualistic illnesses is food sensitivities.

Food sensitivities are distinguished from other types of foodborne disease by the fact that they affect only certain individuals in the population. With food sensitivities, affected individuals experience adverse reactions from eating typical amounts (or sometimes even far less) of a food or food ingredient that most consumers can ingest with impunity.

Many different illnesses occur that fall under the broad definition of food sensitivities. Many consumers and some physicians and other health professionals refer to all of these illnesses as food allergy but, no matter what term is used, it is important to recognise that many different types of illnesses occur as a result of ingesting foods and/or food ingredients on an individualistic basis. These different illnesses can require different diagnostic strategies. In all cases, the most common form of treatment is implementation of an avoidance diet – simply avoiding the food or food ingredient that elicits the adverse reaction. However, the degree of care needed to implement a successful avoidance diet can depend upon the nature of the illness, so it is important for physicians to perform a differential diagnosis and for consumers to know which type of illness that they have.

For the food and related industries, the most important message is that some consumers will not know which type of food sensitivity they experience. These consumers are likely to refer to various food sensitivities as food allergy. To provide the best dietary advice to these consumers, it is important to determine which type of food sensitivity is occurring. It is especially important to recognise when a true food allergy is involved, because avoidance can be difficult due to the very low thresholds that some of these consumers have for the offending food.

Food allergies are abnormal immunological responses to a particular food or food component, usually a naturally occurring protein. Two types of abnormal immunological responses can occur – immediate hypersensitivity reactions and delayed hypersensitivity reactions; both are well documented as occurring in affected individuals upon ingestion of specific foods. Immediate hypersensitivity reactions are IgE-mediated reactions with symptoms ensuing within minutes of the ingestion of the offending food. Delayed hypersensitivity reactions are cell-mediated reactions with symptoms developing 48-72 h after ingestion of the offending food. The role of cell-mediated reactions in food allergies is far less well established; IgE-mediated food allergies, by contrast, are quite well understood.

Food intolerances do not involve abnormal responses of the immune system. Three distinct forms of food intolerances are recognised: anaphylactoid reactions, metabolic food disorders, and idiosyncratic reactions. With a few very noteworthy exceptions (e.g. lactose intolerance), the food intolerances are not well understood. Although these illnesses do not truly fall into the category of food sensitivities, it is important to mention allergy-like intoxications at this juncture. Allergy-like intoxications are often confused with true food allergies because the symptoms are often quite similar. Histamine poisoning is the primary example of an allergy-like intoxication. All consumers are susceptible to histamine poisoning if they ingest sufficient amounts of histamine in their diet. Histamine poisoning most typically occurs on ingestion of foods, especially some fish species (tuna, mackerel, mahi-mahi), that have been subjected to improper, elevated storage temperatures and allowed to spoil.

#### **4. Allergen control in the food industry**

When a food safety issue due to mishandling of allergenic ingredients occurs, everyone in the food processing industry suffers. Consumers depend on food companies to provide safe products. Consumers who must be mindful of the foods they eat because of potential allergic reactions are especially dependent on the industry's ability to identify, process and market foods which are labelled correctly. Food-allergic consumers must avoid the foods that trigger their allergic reactions. Thus, they rely heavily upon the ingredient statements of packaged food products to identify the products which contain their allergen(s).

Food labelling for the presence of allergenic foods/ingredients must identify all foods that intentionally contain the particular food or ingredients derived from that food. However, voluntary labelling for the possible presence of an allergen (e.g. "May contain") should be reserved for situations that potentially represent genuine hazards. In recent years, there has been a proliferation of the use of precautionary allergen statements, which range in wording from "May contain" and "Processed in a facility", to "Made on shared equipment". This increase has limited consumer food choices. Alarming, food-allergic consumers, especially teens, are beginning to ignore precautionary

statements, and taking risks regarding the food they choose to eat. This can lead to trouble for both the consumer and the industry.

An allergen control plan is a critical component in product safety initiatives. For food industries [13] looking to establish a food allergen control plan, there are three key steps:

a) Risk Assessment: Risk assessment involves a hazard analysis by a multifunctional team that includes members from such departments as manufacturing, quality, food safety, sanitation, research and development, and regulatory compliance. Risk assessment helps identify potential sources of food allergens and maps their path through each step of the manufacturing process. Once the path is identified, controls can be put in place in target areas such as reception and storage, scheduling of production runs, variations in production, equipment design and supply and cleaning materials. It is important to periodically review and reassess risk assessments, as new products, formulation changes or vendor changes can change production conditions.

b) Risk Management: The key to successful risk management is developing work instructions and standard operating procedures that control the possibility of unintentional allergen contamination. These procedures and instructions include quality requirements for vendors' ingredients, segregation, production controls, manufacturing scheduling, equipment and plant design, as well as cleaning and sanitation procedures. It is important to validate that these procedures and practices are effective using a science-based approach. In addition, these activities should be routinely reviewed and evaluated for effectiveness. A successful allergen control plan relies on continuous training, clear explanation of procedures and documentation of the existence and effectiveness of the plan.

c) Risk Communication: The next step after assessment and management is communication. If an allergenic food in a plant could be unintentionally found in the finished food product, it is essential this information appears on the food label. Risk assessment can help define the nature of the potential allergen. Is the final product manufactured from ingredients that contain allergens, or is it manufactured on equipment that is in direct contact with allergenic ingredients? This analysis can ensure proper labelling, either in the food ingredients or as a precautionary allergen label. Ultimately, it is important to remember that food allergen control plans require management commitment to succeed.

Continual communication and training increase the safety of manufactured products. Allergen control is but one of the many efforts to prevent and minimise foodborne illness in humans, but the development of and adherence to an effective allergen control plan will go far in protecting allergic consumers and reducing the food manufacturer's risk to reputational and recall costs.

Establishing proper communication between and among customers and food service employees can be one of the first and most important steps in preventing food allergy reactions in restaurants [14,15]. Proper communication among stakeholders should initiate increased attention by food preparation and service staff when serving customers with food allergies. Although there are other food allergy-related publications available, no research has been published regarding food allergy risk communication.

Researchers found that restaurant staff lacked knowledge regarding food allergens in the menu, ways to prevent cross contact and the severity of food allergy reactions [16]. One study from the UK revealed that about 21% of the peanut-free meals that were prepared immediately after peanut-containing meals were contaminated with peanut or peanut protein. Researchers also found that restaurant employees' confidence levels were high even though their knowledge about serving customers with food allergies was not adequate. Specifically, 70% of the respondents in this study felt they could guarantee a safe meal, while 35% thought fryer heat could destroy allergens and 25% thought it was safe to remove allergens from a finished meal [17].

Strict avoidance of food allergens and early recognition and response to allergic reactions are extremely important for individuals with food allergies to prevent fatal food allergy reactions [18]. To prevent potential food allergy reactions, customers with food allergies have used various strategies prior to and while dining out. For example, customers chose restaurants with which they were familiar and where they were known by the staff; avoided establishments and cuisines that are considered high-

risk such as buffets or ethnic restaurants; and checked online menus, ingredients, and allergen information before dining out [19].

Despite these prevention strategies, customers with food allergies have experienced communication challenges when dining out because some restaurant staff did not seem to have knowledge about food allergies, did not understand special requests, and were not aware of the severity of food allergy reactions [6]. There is a lack of legislation or training guidelines focusing on the risk management of food allergies and risk communication-related issues in restaurants. Yet most food handlers perceive the foodservice industry as a low-risk business, which negatively affects their safe food-handling behaviours [7]. Therefore, food allergy risk communication can be used as a tool to reduce the chance of food allergy reactions caused by the mistakes of restaurant staff when serving customers with food allergies [8].

Precisely defined and consistent safety management standards for allergens lead to a consistent and sustainable food safety management in the food industry, but also in the production of gastronomic products. Allergenic foods can be risky in two cases: when they are directly taken into the body alone or as an integral part of a gastronomic product, or by cross-contamination of non-allergenic foods with allergenic ones during the food production process [9].

## **5. Allergen policy**

The allergen control plan must be implemented, audited, enforced and updated continually [20]. Allergen information needs to be regularly updated, especially when new ingredients or different brands of ingredients are introduced to the menu. Staff must be familiar with this procedure to ensure that they can deal with such requests from company. The allergen control plan should address the following activities:

- Supplier monitoring
- Plant traffic flow
- Raw material storage
- Color-coding systems for utensils used with allergens
- Production scheduling
- Cleaning
- Use of rework
- Evaluation of program effectiveness
- Label review policies
- Frequency of plan review
- Documentation and documentation review of activities
- Employee education

## **6. Predictive modelling of allergenic foods**

In the area of risk management of allergenic foods in the food industry, there is an irrational opinion of zero risk tolerance that entails the complete avoidance of any food that is potentially a causative allergen [21]. The risk of cross-contamination by allergens during the food production is present despite the efforts of food producers to comply with all the requirements for applying the principles of good hygiene practice.

Predictive modelling in risk management of allergenic foods is significantly hampered by a poorly defined method food declaration, whereby the zero risk tolerance for allergens induces food producers to use the term “May contain” on food labels [22]. Some stakeholders are not convinced that the new labelling legislation provides sufficient information to allergic consumers [23]. Due to fear of cross-contamination in the absence of accurate precautionary or “May contain” labelling, food allergic consumers are uncertain about product safety, and might not be able to understand or interpret the information on the food labels. For food allergic consumers, unintentional exposure to allergens when

eating outside the home in restaurants and other catering outlets is particularly problematic, as unintentional exposures to problematic allergens can occur.

The need to establish a reliable system of declaring, labelling and marketing of foods has caused a necessary step ahead in science, such as the determination of eliciting doses (ED) of allergenic food ingredients, which vary depending on individual predispositions and geographical determinants. The initial EDs of proteins in allergenic foods were taken as the highest ones found by the research group of Allergen Bureau VITAL scientists in Australia [24]. The VITAL 2.0 program has established reference doses of total allergenic protein intake and defined an action network of risk levels for allergenic foods, calculated by using reference doses and reference quantities of food intaken/portion sizes of gastronomic products (i.e., the ratio of the reference dose and the amount of food intaken or the portion size of the gastronomic product for one meal). By determining the reference doses, an effective basis for communication within the risk management of allergenic foods has been set up, which has enabled detailed identification, characterisation and significantly easier risk management, weighting and selection, i.e. detailed risk analysis for food allergens.

## 7. Conclusion

An increasing number of people are being diagnosed with food allergies. For some, the repercussions could be fatal. The foodservice industry is going to encounter an increasing demand for special meals to cater for those with allergies. Allergenic foods and gastronomic products that contain them are distinguished by specific characteristics compared to other health and safety risks. In the hospitality industry, there is a general lack of knowledge of food allergies, and staff may not be able to respond adequately to requests for non-allergenic foods. However, allergens can be controlled and minimised using careful risk analysis throughout each segment of food chain. The key basis for security management of allergens is good consumer-to-customer and employee-supplier communication and excellent interpersonal communication within each foodservice facility. However, the risks beyond the control of allergen management are undeclared or wrongly declared allergens and unverified allergies. Allergens in foods that are not properly labelled or are highlighted in a less recognisable way can cause significant failures in the safety management system. Another risk which is almost impossible to avoid is an allergic reaction occurring for the first time. These situations require that there is a person in hospitality facilities who is trained to recognise such symptoms and react correctly and in a timely manner. Understanding the similarities and differences in attitudes, knowledge and training with respect to food allergies between hospitality managerial staff and employees would help food industries plan and implement policies and training that best fit both managerial staff and employees.

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# Real-time PCR methods for detecting *Salmonella* spp. in food after different DNA extraction procedures

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**Abstract.** The aim of this paper was to evaluate two real-time PCR (qPCR) protocols for the detection of *Salmonella* spp. in minced meat and chicken neck skin, after DNA extraction using the *InstaTM Gene matrix* (BioRad, USA) and DNA extraction based on thermal cell lysis. The applied molecular methods were sensitive and specific for the rapid detection of *Salmonella* spp. in minced meat and chicken neck skin. The qualitative results were identical regardless of the applied DNA extraction or qPCR protocols. Lower Cq values were achieved after DNA extraction using the *InstaTM Gene matrix*.

## 1. Introduction

*Salmonella* species are one of the main foodborne pathogens [12]. The most common sources of human infections are food products of animal origin, especially pork and poultry meat [3,4,5,6,7]. In the European Union, 91,662 cases of salmonellosis were confirmed during 2017 [14]. In Serbia, 1,850 cases of salmonellosis were diagnosed during 2017, which is 16.4% more cases than in 2016 [13]. The standard method requires at least four days for the detection of *Salmonella* spp. in food. Modern food microbiology demands the implementation of faster methods for the detection of *Salmonella* spp. [2,8]. The qPCR method meets this requirement, but it is still relatively more expensive than the cultural method. The aims of this study were to:

1. Evaluate a modified qPCR protocol for the detection of the *invasion gene (inv A)* [9] and the *tetrathionate respiration gene (ttr)* [11] *Salmonella* spp. in minced meat and chicken neck skin samples and to compare results with the reference method [17].
2. Compare two DNA extraction procedures and determine the effect of using different volumes of BPW pre-enrichments for the DNA extractions.

## 2. Materials and methods

### 2.1. Type of samples

A total of 154 samples (Table 1 and 2) were examined for the presence of *Salmonella* spp. using qPCR methods for detecting the *inv A* and *ttr* genes of *Salmonella* spp. with parallel testing using the reference method [17].



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**Table 1.** Examined food samples

Food category	Natural samples	Artificially contaminated samples
Chicken neck skin	50	30
Minced meat	74	0
<b>Total</b>	<b>124</b>	<b>30</b>

Chicken neck skin samples were artificially contaminated with a reference strain of *S. Typhimurium* (ATCC 14028) at two contamination levels (1-10 and 10-100 cfu per 25 g of sample). Uninoculated samples were used as negative controls.

## 2.2. Isolation of *Salmonella* spp.

The cultural detection of *Salmonella* spp. was conducted using the reference method [17].

## 2.3. DNA extraction

After the sample pre-enrichment in Buffered Peptone Water (Oxoid, UK) for 16-20 h at 34-38 °C, two DNA extraction procedures were applied: DNA extraction based on thermal cell lysis (TL) and DNA extraction using the *InstaTM Gene matrix* (IGM) (BioRad, USA) as we described in our previously published paper [1].

The detection of *Salmonella* spp. was also performed after DNA extraction of pooled pre-enriched test portions obtained by mixing 200 and 300 µL of pre-enrichment of naturally contaminated samples with 800 µL and 1200 µL of pre-enrichment in which *Salmonella* spp. was not detected. The PCR was performed with the addition of 2 or 4 µL of extracted DNA.

## 2.4. Real-time PCR methods

The detection of *inv A* (Protocol *invA*) [9] and *ttr* genes in *Salmonella* spp. (Protocol *ttr*) [11] was performed with the modifications described in our previously published study [1].

## 2.5. Terms and Statistical Analysis

The obtained Cq values were analysed by t-test in *Excel* (Microsoft Corporation, USA). The comparison and interpretation of the results (Table 2) between the reference and alternative methods were conducted in accordance with the ISO 16140 [10].

## 3. Results and discussion

In the presented study, two non-patented qPCR protocols after two different DNA extraction procedures were compared with the reference method [17] for the detection of *Salmonella* spp. Additionally, genomic DNA of *Salmonella* spp. was detected after DNA extraction of pooled pre-enriched test portions. The qualitative results of this study were identical regardless of the applied DNA extraction procedure or the qPCR protocol for the detection *Salmonella* spp. in chicken neck skin and minced meat samples (Table 1). No false negative results were detected. The relative trueness and the sensitivity for both the alternative and reference methods are summarized in Table 2. The results were compared to those of the reference method for a total of 154 naturally or artificially contaminated chicken neck skin and minced meat samples [17].

**Table 2.** Comparison of gene detection results between the reference and alternative methods

Protocol	No of samples	Alternative method	Reference method		SE <sub>alt</sub>	SE <sub>ref</sub>	RT
			R+	R-			
<i>invA</i>	154	A+	PA = 35	PD = 0	100 %	100 %	100 %

<i>ttr</i>	154	A -	ND = 0	NA = 119	100 %	100 %	100 %
		A+	PA = 35	PD = 0			
		A -	ND = 0	NA = 119			

Legend: Alternative method: positive (A+) / negative (A -); Reference method: positive (R+) / negative (R-); Positive Agreement (PA); Positive Deviation (PD); Negative Deviation (ND); Negative Agreement (NA); Sensitivity - alternative method (SE<sub>alt</sub>); Sensitivity - reference method (SE<sub>ref</sub>); Relative trueness (RT).

PCR detection of *Salmonella* genes in the artificially inoculated chicken neck skin demonstrated that the best C<sub>q</sub> values (the lowest C<sub>q</sub>) were obtained using the qPCR protocol for the detection of *ttr* gene, after DNA extraction by IGM (Table 3).

**Table 3.** C<sub>q</sub> values obtained after testing the artificially inoculated chicken neck skin samples

No of samples	Contamination level CFU/25 g	qPCR			
		Protocol <i>invA</i>		Protocol <i>ttr</i>	
		IGM	TL	IGM	TL
5	10-100	17.86	22.25	15.21	19.26
20	1-10	19.14	22.39	17.07	20.18
5	0	No C <sub>q</sub>	No C <sub>q</sub>	No C <sub>q</sub>	No C <sub>q</sub>

The detection of *Salmonella* spp. genes in 10 minced meat (pork) samples after DNA extraction in pooled pre-enriched test portions (Table 4) showed an expected impact on the C<sub>q</sub> values. By comparing the C<sub>q</sub> values after the IGM extraction from 1 ml of BPW with the extraction from 200 and 300 µl of BPW, with the addition of 2 µl of the template, or from 300 µl of BPW with the addition of 4 µl template, the following p values were obtained: 0.004, 0.0185 and 0.4884, respectively. By comparing the C<sub>q</sub> values after TL extraction from 1 ml of BPW with the extraction from 200 and 300 µl of BPW, with the addition of 2 µl template, or from 300 µl of BPW with the addition of 4 µl template, the following p values were obtained: 0.0075, 0.0673 and 0.2380, respectively.

**Table 4.** C<sub>q</sub> values obtained after testing the naturally contaminated minced meat (pork, n=10) using the *ttr* protocol, after IGM or TL extraction from different pre-enrichment volumes

Volume of the DNA used as template (µl)		2	2	2	4
Volume of the BPW used for DNA Extraction (µl)		1000	200	300	300
Mean C <sub>q</sub> ± SD	IGM	24.52 ± 1.26	26.49 ± 1.40	25.98 ± 1.27	24.93 ± 1.33
	TL	25.14 ± 1.54	27.46 ± 1.89	26.63 ± 1.87	26.06 ± 1.82

Statistical analysis of the C<sub>q</sub> values obtained after both extraction procedures showed that after extraction from 300 µl of pre-enrichment, using 4 µl DNA as a template, the results were identical to those obtained after extraction from 1 ml of pre-enrichment with the addition of 2 µl of DNA. Extraction from pooled samples could reduce the cost of a PCR method several times, but for routine application, it is necessary to carry out a validation study in accordance with some of the internationally accepted protocols [10] or implement the procedure defined by the standard for sample preparation [15,16].

The applied molecular methods are confirmed as being sensitive and specific for the rapid detection of *Salmonella* spp. in minced meat and chicken neck skin. The duration of analysis for the qPCR methods is approximately 24 h, in contrast to 4-5 days for the reference method [17]. These methods could be used as screening methods, but the reference method remains irreplaceable for confirmatory purposes.

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# Inhibition of *Staphylococcus aureus* by cinnamaldehyde and its effect on sensory properties of milk

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**Abstract.** The antibacterial activity of cinnamaldehyde was evaluated against *S. aureus* experimentally inoculated ( $10^3$  CFU/mL) in UHT-pasteurized milk, which was treated with different concentrations of the cinnamaldehyde (0.1% and 0.05%) and stored at 4 °C for 12 days. The MIC of cinnamaldehyde was 160 µg/ml. During the storage period, *S. aureus* counts in milk were reduced by 0.35-2.77 log CFU/mL. Significantly greater decreases were observed when cinnamaldehyde was added, regardless of the concentration used, compared with the control. A triangle test showed that panellists could detect the difference between milks with different concentrations of cinnamaldehyde ( $P < 0.01$ ). These results suggest that by adding 0.05% cinnamaldehyde to milk, the safety of the milk can be increased and a pleasant, desirable flavour can be obtained.

## 1. Introduction

Foodborne diseases caused by contamination with *Staphylococcus aureus* and enterotoxin production are still a relevant food safety issue, as the numbers of reported cases and outbreaks continue to increase worldwide. Rich in macro- and micro-nutrients, milk is a convenient medium for *S. aureus* growth. Due to the favourable conditions during storage and preparation, staphylococcal enterotoxin can be produced [1] [2].

The dairy industry is improving processing techniques to prolong the shelf life and ensure the safety of milk and at the same time, meet consumers' needs and demands for attractive and more natural products. In recent years, plant essential oils and their major components have been used to improve the safety, quality and sensory attributes of drinks and food. Milk drinks flavoured with cinnamon, cloves and other



spices have become popular in some countries, including Spain and Latin American countries [3], while in Egypt there is a trend to add these flavouring agents to milk intended for manufacturing different types of dairy products. These plant-based antimicrobials can be used as natural preservatives [4] [5]. Essential oils are low molecular weight liquids, limpid, rarely coloured, volatile mixtures that are lipid soluble and soluble in organic solvents [6] [7] [8].

Cinnamaldehyde is the most abundant component of cinnamon essential oil, which is isolated from bark and possesses a wide spectrum of antimicrobial activity against different microorganisms. Cinnamaldehyde is categorised as GRAS (Generally Recognized as Safe) by the Food and Drug Administration, and previous studies demonstrated this compound could be used in the food industry due to its noteworthy antibacterial activity [9].

The aim of this study was to investigate the antimicrobial effect of cinnamaldehyde in different concentrations (0.05% and 0.1%) against *S. aureus* in UHT-pasteurized milk, as well as its impact on the sensory characteristics of the milk.

## 2. Materials and Methods

### 2.1. Materials and culture

UHT-pasteurized milk containing 1.5% fat was bought from a local supermarket. *Staphylococcus aureus* was from the American Type Culture Collection (ATCC) 25923. Cinnamaldehyde (98% purity) was purchased from Carl Roth, Germany.

### 2.2. Determination of minimum inhibitory concentration

Susceptibility of *S. aureus* ATCC 25923 to cinnamaldehyde was investigated by the broth microdilution method. The broth microdilution method was performed in sterile U-bottom microtitre plates. The inoculum density was set to 0.5 on the McFarland scale, then further diluted 10 times in sterile saline and 5  $\mu$ L of this suspension was inoculated in 0.1 mL of Cation Adjusted Mueller-Hinton Broth (CAMHB; Becton, Dickinson and Company, Sparks, USA) to reach a final inoculum of  $5 \times 10^4$  CFU/well. Cinnamaldehyde was diluted in dimethyl sulphoxide (Serva, Heidelberg, Germany) and added to CAMHB in levels from 2560  $\mu$ g/mL to 1.25  $\mu$ g/mL by two-fold dilution in 96-well microtitre plates. After inoculation, plates were incubated at 37°C for 24 h. The minimum inhibitory concentration (MIC) was the lowest concentration of cinnamaldehyde that prevented visible growth of *S. aureus*.

### 2.3. Milk preparation, storage condition and microbiological analysis

Milk was analysed for *S. aureus* on day 0 in order to determine the presence or absence of this pathogen. Approximately 3 log CFU/mL of *S. aureus* was inoculated into *S. aureus*-free milk. Then, experimentally contaminated milk was divided into thirds. Cinnamaldehyde at different concentrations (0.1% and 0.05%, respectively) was added to the first (C1-0.1%) and second part (C2-0.05%), while the third part (C-control) remained without cinnamaldehyde. For bacterial enumeration, 25 mL of milk was transferred into a sterile Stomacher bag and 225 mL of Buffered Peptone Water (BPW) (Merck, Germany) was added. The contents of each bag were homogenized in a Stomacher blender (Stomacher 400 Circulator, Seward, UK) for 2 min. Serial decimal dilutions were prepared and 0.1 mL of appropriately diluted suspension was plated on Baird Parker agar (Oxoid CM 275, Basingstoke, Hampshire, UK) with egg yolk tellurite emulsion (Oxoid CM 275, Basingstoke, Hampshire, UK) and incubated at 37°C for 24 h according to EN ISO 6888-1 [10]. All milks were stored at refrigerator temperature ( $4 \pm 1^\circ\text{C}$ ) for 12 days and examined on day 0 and on days 3, 6, 9, and 12 of storage. Number of colonies was counted, and results were recorded as colony forming units per ml (CFU/ml).

#### 2.4. Sensory analysis

Sensory analysis was performed according to the ISO standard for triangle tests [11], using the UHT-pasteurized milk with two different concentrations (0.05%, 0.1%) of cinnamaldehyde. The aim of using the triangle test was to determine the sensory differences in the attributes most susceptible to modification after addition of cinnamaldehyde (odour, colour and taste). Two sets of three milks, of which two were identical, were offered to each of 12 semi-trained panellists. The panellists were asked to identify the different milk in each set. Milks were presented in 30 mL volumes, served at room temperature in white plastic cups, and coded using three-digit numbers chosen randomly. Water and bread were served to the panellists to clean the palate between the sets. Results were compared with tables of the minimum number of correct responses required for significant differences in this triangle testing [12].

#### 2.5. Statistical analysis

The bacterial counts (mean±standard deviation of log CFU/mL) were analysed by one-way analysis of variance (ANOVA) and individual counts were compared on a 0.05-level of significance by Tukey's multiple comparison test using GraphPad Prism 6 (GraphPad Software, San Diego, California USA, www.graphpad.com). Data from the triangle tests were analysed by counting the number of correct responses (correctly identified different sample) and the number of total responses. These numbers were compared with critical values found in Table 18 in Baltić [12] to determine significant differences.

### 3. Results and Discussion

Cinnamaldehyde showed good antimicrobial activity with an obtained MIC of 160 µg/ml for *S. aureus*. However, despite the good antibacterial effect *in vitro*, higher concentrations are needed to exhibit antimicrobial activity in food model media due to their interactions with food matrix components e.g. fat and proteins [13]. Gutierrez et al. [14] found that for inhibition of *L. monocytogenes* and *P. fluorescens*, approximately 10-fold higher concentrations oregano or thyme were needed in milk than in their control medium. Thus, in the present study, we used approximately 4- and 9-fold higher concentrations of cinnamaldehyde than the MIC we measured.

Initial *S. aureus* counts ranged between 3.31 (control) and 2.29 (0.1% cinnamaldehyde) log CFU/mL and decreased during storage in all milk groups (Table 1).

**Table 1.** Antibacterial activity of essential oil cinnamaldehyde on *S. aureus* counts (log CFU/mL) in milk during storage at 4°C

Group	Days				
	0	3	6	9	12
C	3.31±0.22 <sup>a</sup>	2.87±0.27 <sup>a</sup>	2.91±0.25 <sup>a</sup>	3.14±0.29 <sup>a</sup>	2.96±0.24 <sup>a</sup>
C1	2.29±0.31 <sup>b</sup>	2.37±0.26 <sup>b</sup>	1.93±0.35 <sup>b</sup>	1.77±0.28 <sup>b</sup>	0.54±0.08 <sup>b</sup>
C2	2.45±0.45 <sup>b</sup>	2.46±0.26 <sup>b</sup>	2.22±0.32 <sup>b</sup>	1.91±0.22 <sup>b</sup>	1.62±0.20 <sup>c</sup>

Mean±SD with different lower-case superscript letters in the same column indicates differences ( $P < 0.05$ ); C – control; C1 – 0.1% cinnamaldehyde; C2 – 0.05% cinnamaldehyde.

This finding can be attributed to low temperature storage below 5°C. Although *S. aureus* can survive freezing, the minimum temperature for growth is about 7°C [15]. At the beginning of the study (day 0), initial significant ( $P < 0.05$ ) reductions of *S. aureus* counts were observed in the groups with added cinnamaldehyde. In the milk with 0.1% cinnamaldehyde, *S. aureus* was reduced by 1.02 log CFU/mL, and in the milk with 0.05% cinnamaldehyde, the count of these bacteria decreased by 0.86 log CFU/mL, indicating the immediate antibacterial effect of cinnamaldehyde. Cinnamaldehyde is an aldehyde which, along with terpenes and phenols, is mainly responsible for the antibacterial effect of essential oils [16].

Being hydrophobic, aldehydes interrupt the microbial cytoplasm membrane due to their influence on the unsaturated fatty acids in the membrane [16,17]. Zhang et al. [18] suggested the mechanism behind the antibacterial effect of cinnamon essential oil containing 92.40% cinnamaldehyde. These authors showed that cinnamon essential oil led to leakage of small electrolytes, causing rapid increase in the electric conductivity and leading to decrease in bacterial metabolic activity. These changes happened within the first hours, which could be linked to the decrease in the number of *S. aureus* on day 0 in our study. The decrease in *S. aureus* numbers slowed down in the milks with added cinnamaldehyde during the first three days of storage, and numbers did not change from those on day 0. After that, *S. aureus* numbers decreased during the following days until the end of the storage.

The *S. aureus* count was lower ( $P < 0.05$ ) in the milks with added cinnamaldehyde than in the control milk during the 12 days of storage. An interesting observation is that although the *S. aureus* count was lower in milk with 0.1% cinnamaldehyde than in the milk with 0.05% cinnamaldehyde, no significant differences ( $P > 0.05$ ) were observed between these two milks during storage except on day 12, when *S. aureus* counts were significantly lower ( $P < 0.05$ ) in the milk with the higher concentration of cinnamaldehyde. At the end of the storage period, the *S. aureus* count was 2.96 log CFU/mL in the milk without cinnamaldehyde, 0.54 log CFU/mL in milk with 0.1% cinnamaldehyde and 1.62 log CFU/mL in milk with 0.05% cinnamaldehyde.

These results indicate that cinnamaldehyde could be effective as an anti-staphylococcal substance in the milk or dairy products. However, regardless of antibacterial activity, in general, the use of essential oils and their major components as food preservatives has been limited due their effect on organoleptic properties of food.

The triangle test is a useful method to compare two samples for which differences, especially in flavour, are difficult to detect [19]. Since the control sample obviously differed, we used a triangle test (12 panellists) to clarify the distinction in odour and flavour under normal lighting conditions between milk with 0.1% or 0.05% cinnamaldehyde added. The results (Table 2) showed that in Set II, where the higher concentration of cinnamaldehyde was different, 75% of the panellists marked their ballots correctly, and this number was the critical value 9 at  $P < 0.01$ . When the milk with 0.05% cinnamaldehyde differed (Set I), only 50% of the panellists gave the correct answer, which was not enough to indicate a significant difference (critical value 8,  $P > 0.05$ ). Having in mind that cinnamaldehyde is a yellow oily liquid, its addition in milk at these concentrations did not affect the milk colour (data not shown). However, on addition of cinnamaldehyde to the milk, in which defects and deviations from the characteristic quality are easy to detect, its strong cinnamon odour and sweet taste [20] led to noticeable changes in the sensory properties. The intensity of characteristic odour was equal in milk with both cinnamaldehyde levels, while the sweet taste was more pronounced at the higher concentration of cinnamaldehyde, which made it easier to distinguish this milk. Additionally, panellists indicated milk with 0.1% cinnamaldehyde was less acceptable than milk with 0.05% cinnamaldehyde.

To the best of our knowledge, there are no available data on cinnamaldehyde use in milk and other dairy products or its effect on sensory quality. However, Olmedo et al. [21] used oregano and rosemary essential oils as a preservative agent in cream cheese and found that the inclusion of these essential oils increased bitterness and sourness that significantly changed cream cheese's typical flavour and aroma. The effect of cinnamaldehyde on meat's sensory properties has been examined in studies where it was supplemented in lamb diets as a feed additive [22, 23].

Considering this lack of data and the many difficulties in introducing novel bioactive ingredients, mostly as preservative agents, in dairy products, [24], the results of the present study point to the possibility of designing a new, acceptable, dairy product by adding cinnamaldehyde into the uniform system of fluid milk.



**Table 2.** Scores obtained in the triangle test (triangle testing for difference) comparing milks with different levels of cinnamaldehyde

	Odour	Flavour
<i>Set I: C1 Vs. C2</i>		
Correct replies	6 (ns)	6 (ns)
Incorrect replies	6	6
<i>Set II: C2 Vs. C1 samples</i>		
Correct replies	9 (P<0.01)	9 (P<0.01)
Incorrect replies	3	3

For n=12 panellists, the number of correct answers to conclude that a perceptible differences exist between samples was 8 (P<0.05), 9 (P<0.01) or 10 (P<0.001); ns = not significant; C – control; C1 – 0.1% cinnamaldehyde; C2 – 0.05% cinnamaldehyde.

#### 4. Conclusion

The results of the present study indicate that addition of cinnamaldehyde significantly reduces the number of *S. aureus* in UHT-pasteurized milk. Taking into account this antibacterial effect and the results of the sensory analysis that showed the obvious difference between milks with higher and lower concentrations of cinnamaldehyde, further research should focus on finding optimal concentrations acceptable to consumers. Also, further studies should be focused on application of cinnamaldehyde under abusive temperature conditions and the antibacterial effect of cinnamaldehyde should be assessed against higher levels of *S. aureus* contamination.

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# Validation and application of a total dietary fiber determination method to meat products

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**Abstract.** This paper presents a modification of the reference method for the determination of dietary fiber in food, its validation using two quality control materials and application to meat products. Dietary fiber is a very important food ingredient with multiple positive effects in human nutrition. In recent decades, efforts have been made to enrich with fiber some foods that do not naturally contain fiber to a significant extent, such as meat products. Fiber content must be declared in accordance with legal regulations, and it is necessary to have reliable methods for determining their amount in food. The results obtained show the described modified and optimized method can be applied to meat products, with significant savings in the preparation time and consumption of reagents.

## 1. Introduction

Dietary fiber is, generally, carbohydrates that are indigestible by the human population. There are several ways to classify this fiber, but the most widely used classification is according to their solubility in water. Insoluble fiber is poorly fermented, while soluble fiber is more easily fermented [1].

More recently, the addition of dietary fiber to meat products is gaining in importance with novel understanding of fiber's role in nutrition and human health aspects. Consumption of dietary fiber has a preventive role in the onset of several diseases. Fiber can act as a protective agent against cardiovascular diseases. It reduces the concentration of LDL in the blood and thus acts as reducing agent for hypercholesterolemia and hyperlipidemia [2-5]. Fiber shows affinity for bile acids and cholesterol metabolites, binding them to the small intestine during digestion and preventing their absorption, resulting in a reduction in blood cholesterol [6]. The fiber binds to water, which has a beneficial effect in the gastrointestinal tract, as the volume of the contents increases, reducing the time food is unnecessarily retained in the colon. Thus, the release of toxins and the emergence of cancer are prevented [7]. One of the most popular dietary fiber roles is the ability to regulate overweight and prevent obesity. Fiber consumption slows down the emptying of the stomach by decreasing the absorption of nutrients [8, 9], and thus, the feeling of satiety is prolonged [10-12].

On the other hand, from the aspect of the food industry, the use of dietary fiber has multiple positive effects. Effects that will be manifested in meat products depend on the type and quantity of fiber or the mixture of added fiber. Some of fiber's properties, for example, to bind water, have a positive effect on food consistency, and since fiber is neutral, it will not change the sensory properties of the product. Fiber also has the ability to bind oils, which is essential for the stabilization of



emulsions [13-17]. However, in the food industry, economic profitability is also important. As the sources of dietary fiber are predominantly agricultural by-products which are relatively cheap, their use is very cost-effective [1].

Dietary fiber in the meat industry is mostly used in boiled sausages, fermented sausages, and minced meat products [18-21]. The recommended daily intake of dietary fiber is regulated and is not the same in all countries. It is believed the daily amount of fiber load should not exceed 28-36 g for adults, with 70-80% being insoluble fiber [1]. The main negative effect of excessive fiber intake in humans is the appearance of diarrhea.

For determination of total dietary fiber (TDF) content in food, the reference AOAC 985.29 [22] method is most often used. The aim of this paper was to examine possible application of this method using the FibreBag system and adequate optimization of this analytical process to meat products.

## 2. Materials and methods

All chemicals were purchased from Sigma-Aldrich (Merck, Darmstadt, Germany). The total dietary fiber assay kit was also from Sigma. All other chemicals and solvents were analytical grade. FibreBags S were from Gerhardt (Koenigswinter, Germany). Quality control (QC) materials T2477QC porridge oats and T2479QC bread crumbs were from Fera Science FAPAS (Sand Hutton, York, UK).

### 2.1. Meat products

TDF in 37 meat products from retail were analyzed by the proposed method. Samples were from different brands, manufacturers and retailers. The group consisted of 15 frankfurters, 10 Parisian sausages and 12 chicken burgers. All products had a declared TDF content. Meat products were defatted and dried prior to analysis. Fat content was utilized in calculating the final result % TDF in the meat products.

### 2.2. TDF determination

Reference method AOAC 985.29 "Total Dietary Fiber in Foods Enzymatic-Gravimetric Method" was optimized and applied to meat product samples [22]. The Gerhardt manual fiber analysis FibreBag system FBS6 was used for digestion.

According to the method procedure [22], TDF is determined on duplicate samples of dried and defatted (if fat content is >10%) material. Foods are cooked with heat stable  $\alpha$ -amylase to induce gelatinization, hydrolysis and depolymerization of starch; incubated at 60°C with protease (to solubilize and depolymerize proteins) and amyloglucosidase (to hydrolyze starch fragments to glucose); and treated with four volumes of ethanol to precipitate soluble fiber and remove depolymerized protein and glucose (from starch). The residue is filtered; washed with 78% ethanol, 95% ethanol, and acetone; dried; and weighed. One duplicate is analyzed for protein and the other used to determine ash. The TDF is the weight of the filtered and dried residue less the weight of the protein and ash.

Taking advantage of FibreBag utilization, further investigations were also performed to optimize the process to digest multiple meat samples simultaneously.

### 2.3. Statistical analysis

Statistical evaluation of validation results was performed in MS Office Excel with Data Analysis ToolPack add-in.

## 3. Results and discussion

### 3.1. Method optimization and validation using QC materials

According to application notes from the manufacturer [23], FibreBags are used to determine TDF in the method's filtration step, before determination of ash and proteins in the residue. The FibreBag method was optimized to digest six simultaneous probes (three samples, each in duplicate), and

considering savings of time and material, was evaluated using two QC materials. Six FibreBags with sample portions approximately 1g were placed in the holder and then subsequently digested in accordance with method procedure [22]. After rinsing with ethanol and acetone, FibreBags with samples were dried for 3 h in an oven and consecutively subjected to ash and protein content determination. This procedure provided six times lower consumption of reagents for digestion, and it reduced the analysis runtime.

Validation results of this method procedure are presented in Table 1. The TDF contents determined were inside the declared limits. Interday repeatability was calculated from three replicas of the same sample materials on three different days. TDF determination in QC material T2477QC showed better matching and lower dispersion than results for material T2479QC.

**Table 1.** Validation results of optimized method (\* results are in %)

Material	Assigned value*	Low*	High*	Determined*	Low*	High*	Repeatability
T2477QC	9.50	6.92	12.09	9.52	9.23	9.71	0.25
T2479QC	6.58	4.79	8.37	5.42	4.82	7.01	0.71

### 3.2. Determination of TDF in meat products

Results of determination of TDF in meat products from retail are shown in Table 2. The TDF contents in the examined meat products were relatively uniform. Slightly greater amounts of TDF were observed in chicken burgers, due to their higher content of vegetables. Uncorrected TDF analysis results showed the fiber content in dried, defatted meat samples was from 2.5 to almost 5 percent.

**Table 2.** TDF in meat products

Meat products	Number of samples	TDF range (%)	Average TDF (%)	Uncorrected range (%)
Frankfurters	15	0.50-0.89	0.69	1.35-3.66
Sausages	10	0.61-0.83	0.71	1.43-3.86
Chicken burgers	12	0.68-1.07	0.88	1.67-4.71

## 4. Conclusion

The proposed modified procedure of the reference AOAC 985.29 method for determination of TDF in food using FibreBags can be satisfactorily employed in analysis of both meat products and fiber-rich, vegetable origin food.

The optimization results showed the time required for analysis is significantly reduced, and the consumption of the digestion reagent is six times lower than in the procedure given by the reference method and method recommended by the manufacturer. The consequences are a cost effective method and a larger number of analyzes completed in less time.

## Acknowledgment

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# Packaging as a tool to improve the shelf life of poultry meat

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**Abstract.** In this paper, recent findings about poultry meat spoilage and packaging systems are discussed. Poultry meat is widely consumed and its consumption has been growing for decades. Raw poultry meat is a highly perishable food with short-term shelf life, and thus, novel strategies for maintaining safety and quality must be applied to meet the demands of the modern consumer. The main cause of meat spoilage is bacterial growth and the goal of this paper is to highlight the importance of GHP (*Good Hygiene Practice*) in the chain of processing, packaging, storage, and distribution of poultry meat in order to avoid economic losses and meet consumer expectations.

## 1. Introduction

According to OECD (*Organisation for Economic Co-operation and Development*) data, consumption of poultry meat has been increasing for decades, and the latest data show it is about 24 kg per capita per year in EU countries [1]. Consequently, production in the poultry meat industry is steadily growing, especially in developing countries. It is estimated that poultry meat will account for the largest share of the growth of meat consumed over the next decade to 2025 [2]. In the total production of poultry meat, broiler meat makes up 75%, followed by turkey and duck meat [3]. Therefore, in a context of increasing consumption and production, ensuring the safety and quality of poultry meat products is an important issue. In order to achieve this goal, more efforts are being invested in packing practices that should improve meat safety and quality, as well as extend the shelf life.

In most European countries, poultry meat is reported as an important source of foodborne infections, with *Campylobacter* spp. being responsible for the highest disease burden followed by *Salmonella* spp. [4, 5]. On the other hand, spoilage of raw poultry meat products is a common cause of withdrawal from the market and causes significant economic losses. Microbial spoilage is actually the most common cause of alterations in food quality [6], and meat spoilage is usually caused by proliferation of microorganisms [7].

Nowadays, it is well known that microbiological safety and preservation of raw meat depends on production hygiene, type of packaging and maintenance of the cold chain during processing, storage, and distribution of the product. These three factors are equally important for obtaining quality and safe products, and they complement each other. In this paper, recent knowledge about the microbiological spoilers and packaging techniques for improving the safety and quality of poultry meat are presented.

## 2. Spoilage bacteria in poultry meat

The shelf life of meat and meat products is the storage time until spoilage, where the point of spoilage can be defined by the maximum counts of certain bacteria, or by the development of an unacceptable





off-odour/off-flavour or appearance [8,9]. When large numbers of microorganisms are present in raw meat, there will be changes such that it becomes unappealing and unsuitable for human consumption [7,10]. Generally, it is assumed that spoilage is caused only by some representative species that develop from the initial microbial association with the meat. Thus, microorganisms present on the surface of the chilled poultry carcass originate from the animals (feathers, feet and bodies), slaughterhouse environment (air, water), equipment surfaces and food handlers, and subsequently contaminate cuts and processed meat products. With respect to others sources, animals are the main source of contamination in processing abattoirs. Various microbiotas are hosted in the digestive tract, lungs, skin and feathers. In relation to the aforementioned, evisceration is the most critical point of carcass contamination, because of the microbiota present at high counts in the digestive tract. Commonly reported genera from freshly cut meat are *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Flavobacterium*, *Psychrobacter*, *Moraxella*, *Staphylococcus*, *Micrococcus*, various genera among the lactic acid bacteria and different genera of the family of *Enterobacteriaceae* [11].

The survival and growth of specific spoilage bacteria are further affected by a diversity of environmental condition. These factors, including meat constituents, temperature, pH, oxygen or carbon dioxide (packaging atmosphere) and competing microbiota are important in maintaining meat quality over time [12]. In the context of spoilage, several bacteria are reported as spoilage bacteria of fresh poultry meat cuts. *Brochothrix thermosphacta*, lactic acid bacteria, *Enterobacteriaceae*, *Pseudomonas* spp. and *Aeromonas* spp. are considered as potential spoilers of poultry meat [13-16], but authors point out that the involvement of these bacteria in spoilage could not be clearly concluded. Spoilage of marinated poultry meat is mainly caused by the growth of several species of lactic acid bacteria, which is influenced by the specific composition of marinades and confirmed by metagenomic analysis [17].

### 3. Poultry meat packaging

In recent decades, consumers' perception of food has changed. Consumer demand for fresh and minimally processed foods inspired food researchers to improve food safety and quality and to increase the shelf life of such products. The cutting and wrapping of meat in paper or waxed paper by butchers upon demand were replaced by store cutting and display of the packages in refrigerated self-service display cases, and now, much meat is packaged in the processing plant and stored and displayed in case-ready forms for both raw chilled and processed meat [18]. In order to meet consumer demands, the packaging of fresh meat and fresh meat products to ensure safety, quality, nutrition and convenience is necessary. Furthermore, the packaging of fresh meat products should endure the stresses of handling, transportation, storage, sale, and consumer contact. Meat safety and quality properties are highly dependent on the applied packaging materials and technologies [10]. Packaging of poultry meat and poultry-based meat products has always been challenging because of the meat's perishable nature due to high sensitivity to spoilage and pathogenic microorganisms [19].

Survival and growth of spoilage bacteria are greatly affected by the gaseous composition of the atmosphere surrounding the meat. The recent methods used in poultry industry include vacuum packaging (VP), modified atmosphere packaging (MAP), controlled atmosphere packaging (CAP), active packaging, smart packaging, etc., which strive to enhance the food safety and quality in an as natural way as possible. Modern meat packaging methods maintain a low microbial load while optimising the sensory quality of a product. It is well known that aerobic storage can accelerate spoilage due to the fast growth of *Pseudomonas* spp., while VP and MAP can favour the dominance of facultative anaerobic bacteria including lactic acid bacteria, *B. thermosphacta* and *Enterobacteriaceae* [11]. However, to improve microbiological quality during the shelf life of the packed product, the initial microbial load should be as low as possible. Although it is not easy to determine the count of bacteria at which bacterial spoilage occurs, most authors set a value of 7 log CFU/g as the limit [20,21]. The storage period to reach spoilage (estimated as the time for total viable counts to exceed 7 log CFU/g) can be prolonged by CO<sub>2</sub>-enriched atmospheres when compared to storage under air. With respect to the temperature of storage and distribution, the shelf life of fresh poultry meat before

spoilage occurrence can be extended from six days under air to 12 and 15 days under MAP with 30% CO<sub>2</sub>–70% N<sub>2</sub> and 70% CO<sub>2</sub>–30% N<sub>2</sub>, respectively [14] or from 5 to 8 days with 30% CO<sub>2</sub>–70% N<sub>2</sub> [22].

#### 4. Conclusion

Packaging has an important role in preserving the safety and quality and prolonging the shelf life of fresh poultry meat particularly when correct conditions of hygiene and temperature during processing, storage, and distribution are respected. Development of novel active and intelligent packaging systems has led to the creation of new barriers for food hazards and presents a new approach to food packaging research. However, the massive use of new technologies depends on the price of their application, which is reflected in the price of the final product.

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# Relationships between broiler final weights and microbiota of certain segments of the intestine

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**Abstract.** Only healthy animals can expect good production results. Gastrointestinal tract (GIT) health is of particular importance in broilers. GIT health has been protected by antibiotics as growth promoters for years. Since their use is forbidden, alternatives are required. One alternative is the use of medium chain fatty acid (MCFA) in broiler nutrition, in order to ensure the health of digestive tract, that is, prevent the activity of pathogenic bacteria, coccidias and viruses. Today, commercial MCFA supplement is used on the market in nutrition of broilers and piglets. Previous experiences of using MCFA in nutrition of broilers suggest that MCFA can be used as a substitute for antibiotics. In the duodenum of experimental broilers (a group of birds fed with added MCFA), the numbers of *Enterococcus* spp. and *E. coli* were significantly correlated with bird weight, but this was not the case in other intestinal segments (ileum or caecum).

## 1. Introduction

Food safety is not complete without including safety of agricultural production, which must be provided despite the growing challenges associated with it, especially the growing world population (population bomb), but also other factors (global warming, environmental pollution, agricultural land shrinkage, etc.). In livestock production, the increase in meat production has been largely based on the selection of animals. The production of beef, pork and poultry is still on the rise. The largest increase in meat production in recent decades was the increase in livestock (pig) production for pork. However, poultry meat production will continue to grow at a higher annual rate than pork, and particularly more than beef. This increase in poultry production is based on economic reasons (fast turnover of capital, fattening lasts for 42 days), increasing demand for this meat, and it being a meat that has no religious restrictions. In addition, poultry is especially appreciated by consumers due to its low fat content, especially in breast meat (about 1%). Today's broiler hybrids have been selected so that the heaviest part of the carcass is the breast. In order to fully exploit the genetic potential of today's most common broiler hybrids (Cobb, Ross, Hubbard), paragenetic factors, such as care, accommodation, health care, and especially nutrition, are very important.

The aim of this study was to examine the significance of correlations between the final weight of fattening broilers and the microbiota of individual segments of the intestine.

## 2. Materials and methods

A detailed description of the method of cultivation, keeping, nutrition of broilers, as well as the settings of this experiment was published in Baltić et al. [1]. For the purpose of this work, broilers were fed in all stages of fattening with added MCFA, i.e., a commercial preparation Aromabiotic®, with an average fatty acid composition of: capric acid ( $2.28 \pm 0.05\%$ ), caprylic acid ( $36.85 \pm 0.03\%$ ), capric acid ( $37.88 \pm 1.60\%$ ) and lauric acid ( $24.50 \pm 0.45\%$ ). Samples for microbiological analyses

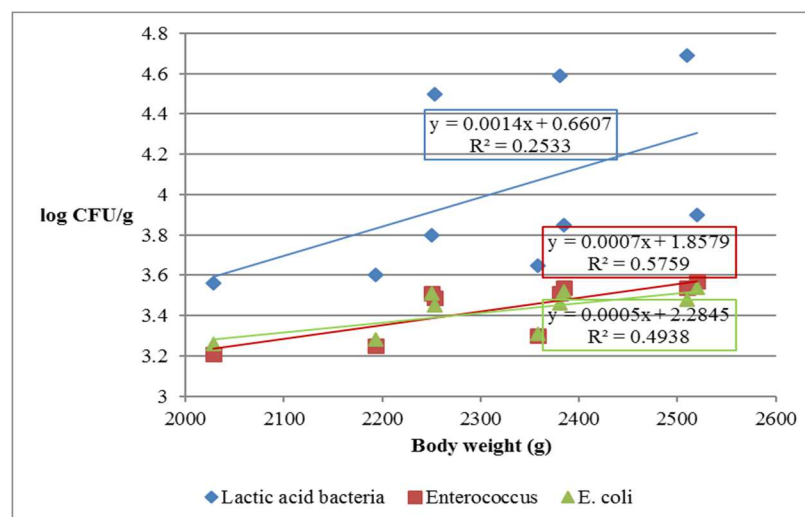


were taken from seven broilers' digestive tracts (duodenum, ileum, cecum) from each group (three groups).

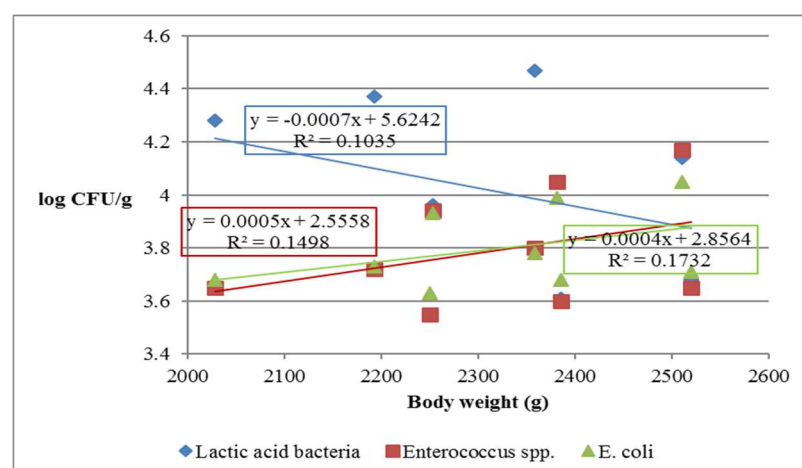
The results obtained were compared by statistical analysis using Microsoft Excel 2010 and GraphPad Prism software, version 7.00 for Windows (GraphPad Software, San Diego, California USA, [www.graphpad.com](http://www.graphpad.com)). Pearson's correlation was used to determine relationships between broiler final weights and microbiota of segments of the intestine (duodenum, ileum, caecum). Differences were considered significant if  $P < 0.05$ .

### 3. Results and discussion

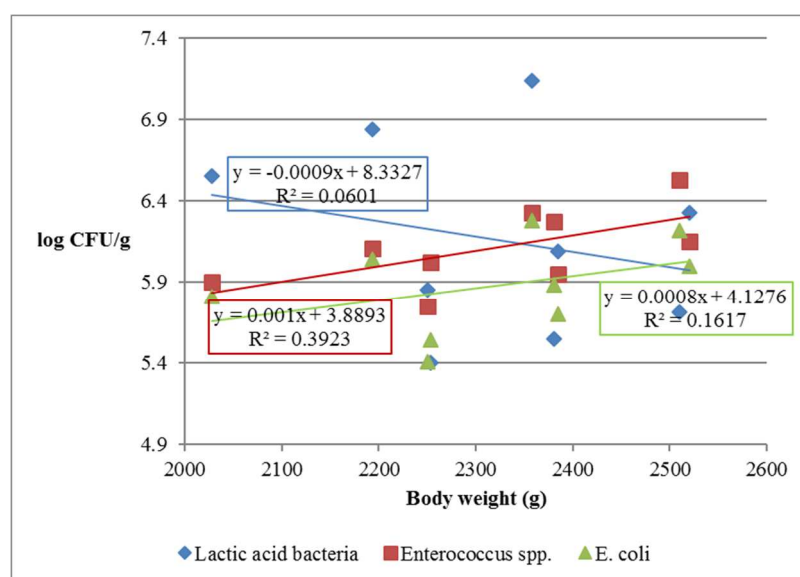
The results obtained for correlations between broiler final weights (g) and determined numbers of isolated bacteria (log CFU/g) from examined intestine segments are present in Fig. 1 (duodenum), Fig. 2 (ileum) and Fig. 3 (caecum).



**Figure 1.** Correlation between broiler final weights (g) and determined number of isolated bacteria (log CFU/g) from duodenum



**Figure 2.** Correlation between broiler final weights (g) and determined number of isolated bacteria (log CFU/g) from ileum



**Figure 3.** Correlation between broiler final weights (g) and determined number of isolated bacteria (log CFU/g) from caecum

Medium and strongly ( $r = 0.759$ ,  $r = 0.703$ , respectively) significant ( $P < 0.05$ ) relationships were found between broiler final weights and numbers of *Enterococcus spp.*, and *E. coli*, respectively, in duodenum. An insignificant medium correlation ( $r = 0.503$ ) was determined between broiler final weights and the number of lactic acid bacteria in the duodenum. A negative, medium correlation between broiler final weights and number of lactic acid bacteria, *Enterococcus spp.*, and *E. coli* ( $r = -0.322$ ,  $r = 0.387$ , and  $r = 0.416$ , respectively), but these correlations were not significant.

The medium relationship ( $r = 0.626$ ) between broiler final weights and number of *Enterococcus spp.* in caecum was not significant. Low correlation dependence ( $r = 0.402$ ) between the number of *E. coli* in caecum and final broiler final weights was not significant. There was no correlation between broiler final weights and lactic acid bacteria in caecum ( $r = -0.245$ ). Table 1 shows correlations between broiler final weights and microbiota of the intestinal segments studied.

**Table 1.** Correlation between broiler final weights and microbiota of intestinal segments

Intestine segments	Intestine microbiota	Correlation coefficient (r)	Interpretation of correlation dependence*	Significance of the difference
Duodenum	Lactic acid bacteria	0.503	Medium	ns
	<i>Enterococcus spp.</i>	0.760	Strong	$p < 0.05$
	<i>E. coli</i>	0.703	Medium	$p < 0.05$
Ileum	Lactic acid bacteria	-0.322	Medium	ns
	<i>Enterococcus spp.</i>	0.387	Medium	ns
	<i>E. coli</i>	0.416	Medium	ns
Caecum	Lactic acid bacteria	-0.245	No relationship	ns
	<i>Enterococcus spp.</i>	0.626	Medium	ns
	<i>E. coli</i>	0.402	Weak	ns

Legend: ns - not significant; \*Source: Colton [2]

In feeding of poultry, it is especially important to preserve the health of gastrointestinal tract (GIT), especially of young animals/birds. Without a healthy GIT there is no healthy animal, feed is poorly

used and production results are unsatisfactory [3]. The harmful agents (bacteria, parasites, toxins, etc.) in GIT are sourced from the environment in which the animals are located (air, equipment) [4], feed and water. The functions of the GIT are to protect the animal from harmful agents (infectious and non-infectious), transporting food ingredients through it, digesting them, and then absorbing nutrients and energy. The GIT is simultaneously a metabolic and immunological organ [5]. Finally, in GIT there are numerous types of bacteria, and according to some data, there are 640 different types of bacteria and over 20 different hormones [6]. The GIT eliminates harmful and non-immaterial food ingredients. Feed can greatly affect the integrity of intestinal mucosa and microbiota of GIT. Among the pathogenic bacteria in the GIT, the most commonly found are *Campylobacter*, *Salmonella*, *Clostridia*, *E. coli*, *Staphylococcus* [7,8,9,10], and among the protozoa, *Eimeria* spp., [11]. In GIT there are also useful bacteria: *Lactobacillus* spp., *Bifidobacterium* spp., *Enterococcus* spp. For a healthy GIT is important to maintain eubiosis, i.e., a balanced state of the pathogenic and useful microbiota [12,13].

Over 50 years in broiler feeding, antibiotics were used as growth promoters or for prevention of adverse actions of pathogenic bacteria in the GIT. After it became clear that bacterial resistance is an increasing public health problem, the use of antibiotics as growth promoters was prohibited in animal nutrition in the EU in 2006 and then in most other countries [14,15,16]. Since the ban on the use of antibiotics in animal nutrition, alternatives have been sought (probiotics, prebiotics, phytogens, additives, enzymes, short-chain and medium-chain fatty acids and other food supplements). Today, more attention is paid to strategies that would stimulate the strengthening of the immune system in animal nutrition [17,4,18,19,20]. For this purpose, amino acids, vitamins, microelements and linoleic acid are used [21,22,23,24,25,26,20,27,28,29,30,31]. In the Cobb Brochure Guide, it is recommended that 1% linoleic acid be used in all three phases of production [32]. MCFAs improve the production results of broilers in fattening (higher final mass, higher growth, better food conversion), and improve lean meat production (higher carcass weight, higher breast mass and higher weight of drumstick with thigh), as well as meat quality parameters (less fat in meat breasts).

MCFAs affect digestive tract microbiota, including coccidias, viruses and, thus, protect the health of broilers. These fatty acids integrate into the cell membrane or enter the cell in a non-desiccated form, especially at a low pH, thereby adversely affecting metabolism of bacterial cells [33].

The effectiveness of the bactericidal effect of MCFA depends on the amount of added MCFA, so the higher added amount the more pronounced is the effect. The reason for less antibacterial activity in lower GIT sections could be because MCFA is already very efficiently absorbed in the duodenum, and so is less available for absorption in the jejunum, meaning its effect here is less pronounced. However, if 3% MCFA is used, there can be a reduction in the number of bacteria [7]. Reduction of potential pathogens in competition with nutrients is significant because pathogens can damage the cellular epithelium of the GIT, which is manifested by reduced nutrition absorption, especially in the small intestine. On the other hand, some bacteria have a positive effect in the digestive tract because they have the ability to ferment and create, for example, butyrates which promote the function of enterocytes and the immune system [34]. It has been suggested that MCFA also have an inhibitory effect via *Lactobacillus* [12], but there are opposite opinions [35].

The GIT of broilers makes up a complex microbiota system that plays important roles in the digestibility and absorption of food, the development of the immune system and the health of animals, and the exclusion of pathogenic activity [10]. Previous studies have shown that nutrition of broilers affects the microbiota of the GIT, or its diversity.

Due to numerous conditions related to the environment in which broilers are cultivated, the microbiota of the GIT can be very different and often changes. The microbiota of the GIT can contain pathogens in large numbers, and this can badly affect the immune system and production results. The potential changes in the number of bacteria is best illustrated by the fact that in a 35-fold increase in bacterial in numbers in the microbial ileum, lactobacilli accounted for over 90% of the increase [10,5].

Coccidiosis is the most economically most important livestock disease in the world and is the most important health problem of its kind [36]. It is believed that coccidiosis losses are two billion dollars in poultry production annually (death, treatment, prevention). In India, in broiler cultivation, 95.6% of

the total losses is associated with coccidiosis-related losses. There are seven species of coccidiosis in chicken: *Eimeria acervulina*, *Eimeria brunetti*, *Eimeria mitis*, *Eimeria necatrix*, *Eimeria pracox* and *Eimeria tenella*, while *Eimeria tenellais* is the most common cause of coccidiosis. Over the last forty years, several different strategies of their control have been developed. The incidence of coccidiosis is significantly influenced by the season and it is most common in the autumn (45,12%), then the summer (30,84%) and the spring (23,81%), and least prevalent in winter (20,29%) [36].

#### 4. Conclusion

In the duodenum of the experimental broilers (a group of birds fed with added MCFA), the numbers of *Enterococcus* spp. and *E. coli* were significantly correlated with bird weight, but numbers of lactic acid bacteria were not. No significant correlations were found between the numbers of bacteria (*Enterococcus* spp., *E. coli* or lactic acid bacteria) determined in other, lower intestinal segments (ileum or caecum), where medium or weak insignificant correlations with bird weight were mostly found.

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# Process hygiene of pig carcasses in one large-scale slaughterhouse in the west of Serbia, during 48 months

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**Abstract.** This study was conducted to determine microbial contamination of pig carcasses during four years in one slaughterhouse. The numbers of total viable counts and *Enterobacteriaceae* and the presence/absence of *Salmonella* spp. are the process hygiene criteria for pig carcasses. We collected 240 samples from April of 2015 to April of 2019, with swabs being continually taken from the carcasses of pigs every month for 48 months in slaughterhouse in the west of Serbia. Over 48 consecutive months of testing, *Salmonella* spp. presence was detected on 1.67% of the pig carcasses, while the determined mean numbers of *Enterobacteriaceae* were  $0.18 \pm 0.37$  log CFU/cm<sup>2</sup>, and the mean total viable count of aerobic bacteria was  $1.88 \pm 0.85$  log CFU/cm<sup>2</sup>. The process hygiene criteria results for the tested pig carcasses showed that for total viable count of aerobic bacteria, 95.35% of carcasses fell into the satisfactory process hygiene group, while 4.17% belonged to the acceptable group. *Enterobacteriaceae* numbers showed 97.90% of the tested pig carcasses belonged to the satisfactory process hygiene group, and 2.10% of carcasses belonged to the acceptable group.

## 1. Introduction

Meat consumption is increasing worldwide due to rapid population growth, urbanization, changing consumer preferences and income growth. Global meat consumption increased by 58% during the past 20 years and in 2018, reached 360 million tonnes [1]. That has resulted in increased concerns and challenges, above all in the field of meat safety and hygiene. To date, the best approach to food safety is a preventive approach, by managing food production from primary production to the consumer. The main responsibility for food safety lies with the Food Business Operators (FBO), who define and implement appropriate measures for good hygienic and manufacturing practice, as well as other procedures based on Hazard Analysis and Critical Control Point (HACCP) principles, in order to achieve the food safety objectives defined in the food regulations. The presence of some microbial indicators is a consequence of direct or indirect contamination of the food with fecal material [2]. The numbers of total viable counts (TVC) and *Enterobacteriaceae* (EC) and the presence/absence of *Salmonella* spp. are the process hygiene criteria for pig carcasses.

During the last few decades, infections with *Salmonella* spp. have been recognized as a major hazard to humans in most developed countries, primarily through contaminated food of animal origin. The genus *Salmonella* covers more than 2400 different serotypes, and although all serotypes must be considered as potential human pathogens, only a limited number of serotypes is attributed as a cause of infection in humans and animals. Although *Salmonella* can survive for long periods in the



environment [3], it is assumed carrier animals are the major source of infection for both animals and humans.

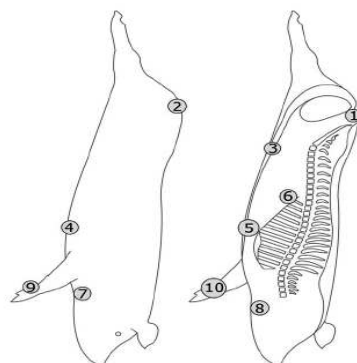
Pig carcasses contaminated with *Salmonella* cannot be recognized during the current veterinary inspection after slaughter. Good manufacturing principles are important to prevent cross-contamination of carcasses during the slaughter process [4]. Cross-contamination in the slaughterhouse is also a big problem from the aspect of meat safety [5], which is confirmed by the increased prevalence of *S. enterica* from farm to slaughterhouse. Furthermore, the contamination/infection of pigs with *Salmonella* spp. can occur at any point from the farm to the slaughterhouse, although it should be emphasized that the slaughterhouse has an important role in this process. The surfaces of the lairage and stunning box are almost always contaminated with *Salmonella*, and these surfaces can be sources of cross contamination, ultimately increasing *Salmonella* prevalences on carcasses on the slaughter line [6]. Operations at the point of slaughter can also have an effect on pig carcass contamination with *Salmonella* [7].

*EC* are very widespread in the environment, and they are also an integral part of the gastrointestinal microbiota of humans and animals. One of the most important places for contamination of pig skin with enterobacteria is the stunning box, which each pig touches. There is also a high risk of meat contamination with gastrointestinal tract contents during pig evisceration. This evisceration is the processing step that most contributes to bacterial contamination on carcass surfaces, because afterward, there is no primary treatment that could reduce the number of bacteria. The technology of pig skin removal after slaughter also carries a high risk of contaminating carcasses/meat with enterobacteria [8]. Moreover, any inadequate procedures during technological operations on the slaughter line can lead to contamination of pig carcasses [9].

The aim of this study was to follow the process hygiene of pig carcasses in one large-scale slaughterhouse during a period of four years. Monitoring hygiene in the slaughterhouse was conducted through process hygiene examinations of pig carcasses and validation of the HACCP system according to the self-control plan of this FBO.

## 2. Materials and Methods

Every slaughterhouse should have a self-control plan specifying time and frequency of sampling, which is regulated according to the: slaughter practice for each animal, design of risk-based process control assurance or harmonized monitoring programs, production volume and the epidemiological status of the area from which the animals originate. The numbers of microorganisms on carcasses were determined according to standard methods [10]. In this study, we used the non-invasive swab sampling method. The swab method is the preferred method for carcass sampling according to HACCP requirements for European Union slaughterhouses [11]. The carcass sites from which samples are taken must be described in the self-control plans, edited by the FBOs. However, since the purpose of this study was to examine those carcass sites where the probability of contamination was the greatest, the recommended standard sampling sites on pig carcasses were used in this study, as shown in Figure 1 [10].



**Figure 1.** Suitable sites for taking samples from pig carcasses [10]. Sites are: 1) Pelvic channel internal, 2) Pelvic channel external, 3) Abdominal, 4) Xiphoid external, 5) U Xiphoid internal, 6) Pillar of diaphragm, 7) Submaxillary external, 8) Submaxillary internal, 9) Forefoot external aspect, 10) Forefoot internal aspect

### 2.1. Process hygiene criteria for pigs

The microbiological criteria for production process hygiene control of pig carcasses were: TVC [12], EC count [13] and the presence/absence of *Salmonella* spp. [14]. Regulations in the EU (No. 2073/2005) [15] and in Serbia [16-18] prescribe limits for process hygiene test results for pig carcasses (Table 1).

**Table 1.** Process hygiene criteria for pigs – non-destructive sampling method [15-18]

Microorganisms	Sampling plan		Limits		Analytical reference method	Stage where the criterion is applied	Action in the case of unsatisfactory results
	N	c	m	M			
Total Viable Counts			3.3 log CFU/cm <sup>2</sup>	4.3 log CFU/cm <sup>2</sup>	ISO 4833		Improvements in slaughter hygiene and review of process controls
EC			1.3 log CFU/cm <sup>2</sup>	2.3 log CFU/cm <sup>2</sup>	ISO 21528-2	Carcasses after dressing but before chilling	Improvements in slaughter hygiene and review of process controls, origin of animals and of the biosecurity measures in the farms of origin
<i>Salmonella</i>	50	3 (5)*	Absence in the area tested per carcass		EN/ISO 6579		

\*[16]; n=number of units comprising the sample; c = number of sample units giving values between m and M.

### 2.2. Samples from slaughterhouse

A total of 240 swabs from randomly selected pig carcasses were collected in one slaughterhouse in Kolubara district, West Serbia. This study lasted for a period of four years, from April 2015 to April 2019. Five samples were collected once a month. Swabs were taken on the slaughter line after the final washout before chilling, in order to monitor compliance with the process hygiene criteria. Process

hygiene was followed and compared with Serbian regulation [16], and followed up by comparison with new Serbian regulation [17] from its date of validation, August 2018.

### 2.3. Statistical analysis

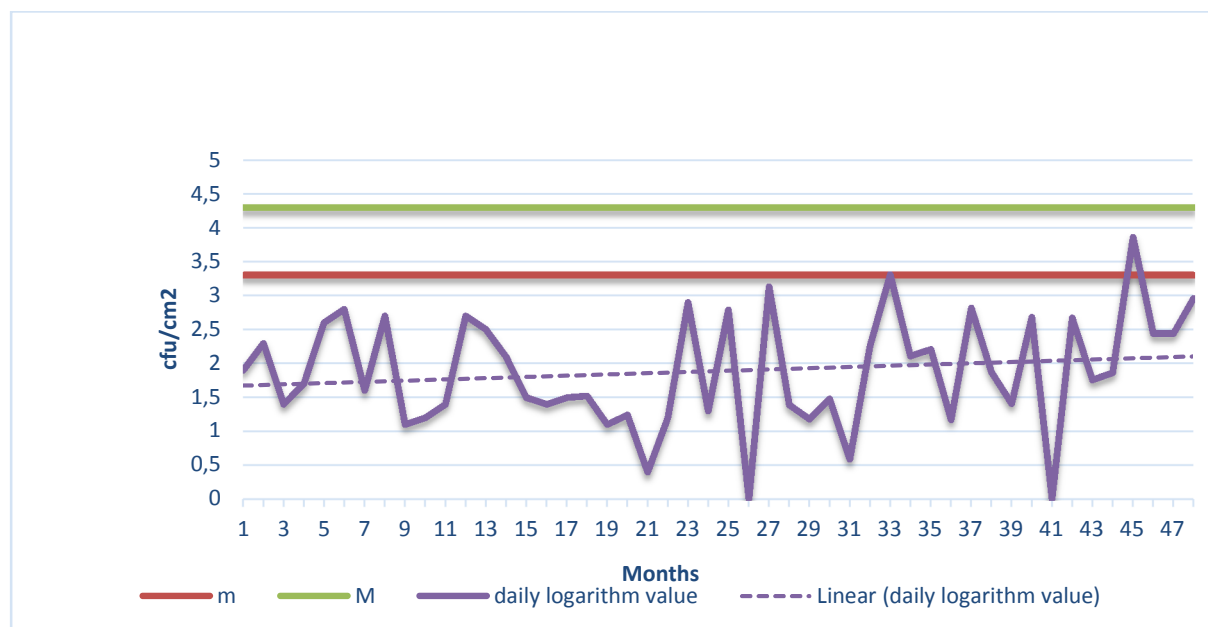
Statistical analysis of the results was conducted with Microsoft Office, Excel program 2016 and GraphPad Prism version 7.00 software. Firstly, the average logarithm value of TVC and *EC* counts for each carcass was calculated (based on previously transformed log values of these bacterial counts for each of four corresponding sites on each carcass), and then the average daily logarithm was calculated. The results were expressed as the mean  $\pm$  standard deviation. The average daily logarithm of *Salmonella* spp. was not calculated, taking into account the regulatory requirement defining only the absence or presence of *Salmonella* spp.

## 3. Results and Discussion

Levels of TVC on the pig carcasses ranged from undetected to 3.86 log CFU/cm<sup>2</sup>, while *EC* levels ranged from undetected to 1.86 log CFU/cm<sup>2</sup>. *Salmonella* spp. were detected on 1.67% of carcasses, while the mean number of *EC* on the carcasses was 0.18 $\pm$ 0.37 log CFU/cm<sup>2</sup>, and the mean TVC of aerobic bacteria was 1.88 $\pm$ 0.85 log CFU/cm<sup>2</sup>.

### 3.1. TVC numbers and trend

The results of process hygiene testing on pig carcasses in this slaughterhouse showed that for TVC, 95.38% of carcasses fell into the satisfactory process hygiene group (equal to or less than 3.3 log CFU/cm<sup>2</sup>), while 4.17% belonged to the acceptable group (3.3-4.3 log CFU/cm<sup>2</sup>) (Figure 2). Results reported previously [19] were similar to the results in our study, as 97% of the carcasses in that study fell into the satisfactory group and 3% fell into the acceptable group. The linear trend for the mean daily TVC on the pig carcasses, which increased over the 48 months, is shown in Figure 2.

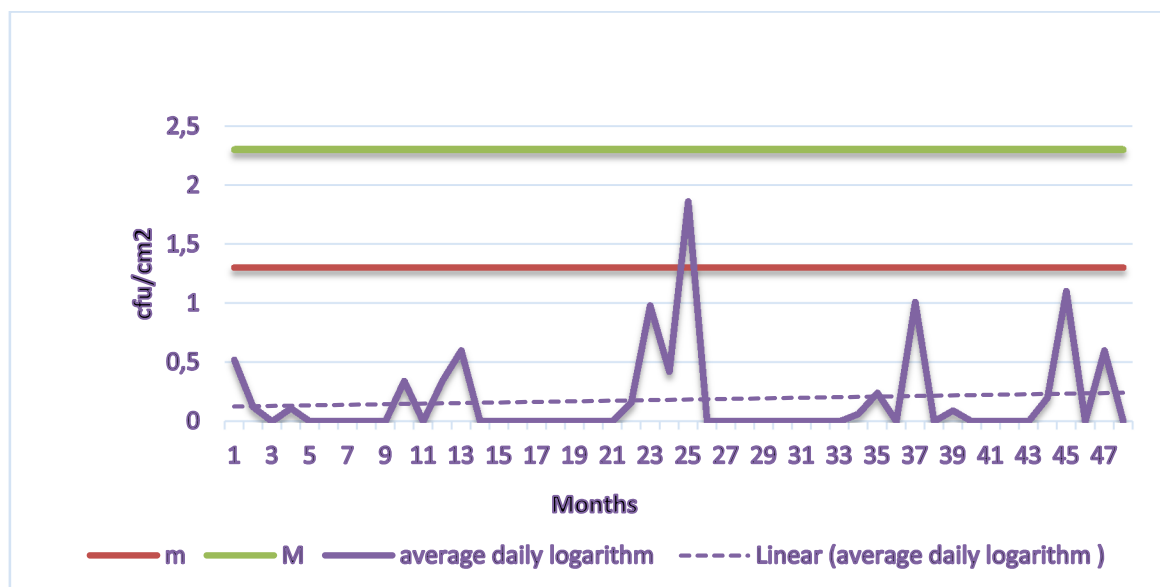


**Figure 2.** Trend analysis of total viable count for pig carcasses 2015-2019

### 3.2. Enterobacteriaceae numbers and trend

*EC* numbers on pig carcasses in the slaughterhouse were such that 97.9% of tested pig carcasses belonged to the satisfactory process hygiene group (equal to or less than 1.3 log CFU/cm<sup>2</sup>) and 2.1% of carcasses belonged to the acceptable group (1.3-2.3 log CFU/cm<sup>2</sup>) (Figure 3). Similar results were

found by Milojević *et al.* [19], who reported that 99% of tested pig carcasses belonged to the satisfactory group and 1% belonged to the acceptable group. Figure 3 shows the slightly increasing linear trend for *EC* over our 48 month study.



**Figure 3.** Trend analysis of *Enterobacteriaceae* for pig carcasses 2015-2019

### 3.3. *Salmonella* spp. presence/absence

The presence of *Salmonella* spp. was detected on 4 of the 240 pig carcasses examined. The regulatory limit for detection of *Salmonella* spp. is 3 times in 50 samples. These current results differ from the results of Mrdovic *et al.* [20], who carried out research in another district in Serbia, but detected the presence of *Salmonella* spp. only twice during a period of six years (2011-2016). Because *Salmonella* spp was detected at the slaughterhouse, the origin of animals and biosecurity measures on the farms of origin had to be checked, process controls reviewed, and slaughter hygiene improved.

## 4. Conclusion

We conclude that more than 95% of tested pig carcasses at slaughter in this FBO's premises had satisfactory process hygiene indicators, *EC* (97.9%) and TVC (95.38%).

The FBO was required to perform corrective actions because of the presence of *Salmonella* spp. on the pig carcasses at slaughter. The FBO had to improve slaughter hygiene and review measures for process control, check the origin of the animals and examine biosecurity measures in the farms of origin according to their self-control plan.

The process hygiene indicators and microbial quality of meat for consumption are closely related to public health, and so the FBO must have proper control over the production process. Linear trends of the process hygiene data for both TVC and *EC* showed increasing numbers of both bacteria indicators of process hygiene on the pig carcasses over time.

FBOs should more respectful of their requirements to fulfill good manufacturing practice (GMP) and good hygiene practice (GHP) measures and improve production hygiene in the slaughterhouse. The pre-requisite GHP and GMP programs must work effectively before HACCP is applied. HACCP is the best system currently available for maximizing the safety of meat and meat products, as well as food in general, and requires the FBO to proactively recognize, control and/or eliminate relevant hazards that could compromise product safety.

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# Lactic acid bacteria isolated from sremska sausage using molecular methods

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**Abstract.** Sremska sausage is a traditionally fermented dry sausage, which is produced in northern Serbia (Vojvodina). It is made from pork with the addition of back fat and natural spices. The entire manufacturing process lasts for 21 days. The goal of this study was to create a collection of lactic acid bacteria isolated during sausage fermentation and identify them using molecular methods. A total of 50 isolates from different stages of fermentation were identified by molecular methods. *Ln. mesenteroides*, *Pediococcus pentosaceus* and *Lb. sakei* are the predominant microorganisms in the sremska sausage studied.

## 1. Introduction

Sremska sausage is a typical dry fermented sausage produced in northern Serbia (Vojvodina), especially in the area of Srem. It is traditionally produced in households as well as in meat plants. It is made from pork (shoulder), back fat and spices such as sweet and hot ground pepper and garlic and filled into small pork intestine. Sremska sausage is characterized by a recognizable and characteristic flavour of smoke and the added spices.

Traditional fermented sausages with a specific geographical origin have unique sensory characteristics and are generally of high quality [1]. Sensory properties of traditionally fermented sausages depend on the type of meat, salt, spices, additives, smoking intensity, temperature of fermentation, duration of drying and metabolic activity of the microbiota.

During sausage fermentation, different groups of microorganisms possessing specific biochemical potentials contribute to the sensory profile of the final product [2]. The microbial activity and interaction are key factors for the final quality characteristics of fermented sausages [3]. In order to protect the traditional aspect of sausage manufacturing, it is essential to understand the microbial diversity and to select autochthonous starter cultures that could be used in the production of innovative foods with a geographical origin [4]. Fermentation with autochthonous starter cultures allows for controlled production with lower safety risks than in products obtained after natural fermentation [5]. Studies of the microbiota in traditional meat products have revealed considerable microbial diversity [6].

Lactic acid bacteria (LAB) play an important role in meat preservation and fermentation and are considered as a technologically fundamental group of microorganisms. They are able to decrease the pH by lactic acid production, produce bacteriocins which prevent the growth of pathogenic and spoilage microorganisms, provide diversity of sensory properties by modification of raw material,



contribute to the development of flavour, colour and texture and improve the safety, stability and shelf life of meat products [7,8].

The main LAB that have been isolated from fermented sausages belong to the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Weissella* and *Enterococcus* [9,10]. The most commonly identified LAB species in traditional fermented sausages are *Lb. sakei*, *Lb. curvatus* and *Lb. plantarum* [10,11].

In order to create a collection of autochthonous natural isolates of LAB as a future basis for the production of starter cultures with a geographical origin, LAB strains were identified and characterized by molecular methods.

## 2. Materials and Methods

### 2.1. Sremska sausage

Sremska sausage was produced by the traditional method in a family household in Srem (northern Serbia). Sremska sausage was composed of pork (shoulder) and back fat in the percentage ratio 70%:30%. Meat and fat were ground to the size of 8 mm and mixed with nitrite curing salt (2.5%), sucrose (0.33%) and spice mixture (0.25%; composed of sweet and hot red peppers, black pepper and garlic). Prepared stuffing was filled into small pork intestine (diameter of 34-36 mm). Sausages were cold smoked for 3 days. The entire manufacturing process (smoking, fermentation/ripening and drying) in this traditional process lasted for 21 days.

### 2.2. Microbiological investigation

Sausage samples for microbiology examinations were taken on days 0, 2, 4, 7, 14 and 21. The experiment was repeated three times. Three samples at each step of sampling were collected and used for analysis. Each sample weighing 25 g was homogenized in 225 ml of MRD (Oxoid, UK) in a stomacher (AES, France) for 90 s. Serial dilutions (10-fold) were plated onto MRS agar (Oxoid, UK) in duplicate, and incubated for 48 h at 30°C under microaerophilic conditions. From each plate, single colonies were randomly picked and streaked on new agar plates in order to obtain pure cultures. The LAB isolates from MRS agar were checked by Gram staining and catalase reaction. A total of 50 Gram-positive and catalase-negative isolates were further identified and characterized by molecular methods.

Total DNA from LAB was extracted from a single colony by using the DNeasy Blood and Tissue Kit (Qiagen GmbH, Germany) according to the manufacturer's protocol for Gram-positive bacteria. PCR was performed in a final volume of 50 µL containing 1× PCR buffer (10× PCR buffer: 500 mM KCl, 100 mM Tris-HCl, 0.8% Nonidet P40), 2.5 mM MgCl<sub>2</sub>, 10 µM dNTP, 200 nM of each primer, 1 U of Taq polymerase (Fermentas, Lithuania) and 100 ng of DNA template. The DNA was amplified in a thermal cycler (Techne, UK) using primers P1V1 (GCGGCGTGCCCTAATACATGC) and P4V3 (ATCTACGCATTTCACCGCTAC), complementary to the V1-V3 region of the 16S rRNA, 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 42°C, 2 min at 72°C and the final extension of 5 min at 72°C. PCR products were purified by QIAquick PCR purification kit (Qiagen, Germany) and sent for sequencing to IIT Biotech (Bielefeld, Germany). The BLAST algorithm was used to determine the most related sequence relatives in the NCBI nucleotide sequence database (<http://blast.ncbi.nlm.nih.gov>).

## 3. Results and Discussion

**Table 1.** Lactic acid bacteria isolated from sremska sausage and identified by molecular methods

Day of fermentation	16S rRNA gene sequencing
0	<i>Ln. mesenteroides subsp. mesenteroides</i> (5 isolates)

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	<i>Pediococcus pentosaceus</i> (3 isolates)
	<i>Lc. lactis subsp. lactis</i> (2 isolates)
	<i>Weissella viridescens</i>
	<i>Ln. mesenteroides subsp. mesenteroides</i> (3 isolates)
2	<i>Ln. gasicomitatum</i>
	<i>Pediococcus pentosaceus</i> (2 isolates)
	<i>Ln. mesenteroides subsp. mesenteroides</i> (6 isolates)
4	<i>Pediococcus pentosaceus</i> (2 isolates)
	<i>Ln. gasicomitatum</i>
	<i>Ln. mesenteroides subsp. mesenteroides</i> (4 isolates)
7	<i>Lb. plantarum</i>
	<i>Lb. sakei</i> (2 isolates)
	<i>Ln. mesenteroides subsp. mesenteroides</i> (4 isolates)
	<i>Pediococcus pentosaceus</i> (2 isolates)
14	<i>Weissella viridescens</i>
	<i>Lb. curvatus</i> (2 isolates)
	<i>Lb. sakei</i> (2 isolates)
	<i>Lb. sakei</i> (2 isolates)
21	<i>Ln. mesenteroides subsp. mesenteroides</i> (2 isolates)
	<i>Lb. alimentarius</i>
	<i>Weissella viridescens</i>

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The most dominant microorganisms in sremska sausage were *Ln. mesenteroides*, *Pediococcus pentosaceus*, *Lb. sakei*, *Lb. curvatus*. Besides them, *Lc. lactis subsp. lactis*, *Weissella viridescens*, *Ln. gasicomitatum*, *Lb. plantarum* and *Lb. alimentarius* were also identified, although in lower numbers.

The dominant presence of *Ln. mesenteroides* found in sremska sausage is in accordance with results obtained for Petrovská klobása [12], in which *Ln. mesenteroides* and *Lactobacillus* constitute the majority of the microbiota. Some of the *Leuconostoc* strains isolated from fermented sausages play an important role in the flavour development and could also exhibit strong antimicrobial activity [13,14].

The prevalence of *Pediococcus pentosaceus* isolated from our sremska sausage is in accordance with results obtained for Iberian dry sausage [15] and Italian fermented sausage [16]. The predominant presence of *Lb. sakei* is in accordance with results obtained by several authors [7,11,17,18]. These species are the most well-adapted lactobacilli to the fermented sausages environment [11].

#### 4. Conclusion

The most dominant LAB during the whole fermentation of this traditionally produced sremska sausage are *Ln. mesenteroides*, *Pediococcus pentosaceus* and *Lb. sakei*. At the beginning of the production, *Ln. mesenteroides* and *Pediococcus pentosaceus* prevails. From day 7 until the end of production, the dominant strain is *Ln. mesenteroides*, followed by *Lb. sakei* and *Lb. curvatus*. The isolated strains of LAB could be used as starter cultures for fermentation of traditional dry sausages.

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# Assessment of sensory and chemical parameters of tea sausage

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**Abstract.** In the former Yugoslavia, the production of tea sausage started just over half a century ago. This type of sausage is mostly industrially produced, which means the quality of this product is not standardised, but it is acceptable to the majority of the population. In this study, the sensory proprieties and chemical characteristics of tea sausage were examined. Two types of tea sausage were made, which differed depending on the percentage of different categories of pork meat, while the amount of beef meat, solid fat tissue and other ingredients were the same. The results obtained show the average scores for all tested sensory properties almost equal. Chemical results show that tea sausage-group 1 had higher protein content (27.96%) than tea sausage-group 2, which had 25.25 % protein in meat, while the content of collagen in meat protein was similar in the two sausage groups. Moisture in these sausages was less than 35%, while the values of other parameters were similar. This study demonstrated that different quantity and quality of pork meat could influence the sensory properties and chemical composition of tea sausage.

## 1. Introduction

Fermented sausages are much appreciated, high-quality products of the meat industry. There are many historical accounts about the production and consumption of sausages in ancient civilizations, going back thousands of years. However, there is no specific date when the first sausage was produced, because this dates from the period before written history [1]. The first records of fermented sausages are from 3000 BC, but more information dates from China and the Mediterranean region from about 2000 BC [2]. During the Middle Ages, great migrations led to the mixing of different cultures and customs, and therefore, knowledge of food conservation was transmitted worldwide more rapidly. After the Second World War, development and modernization of product technology and equipment for fermented meat products continued [3]. The available literature provides information on authentic of fermented sausage production with special emphasis on microclimate conditions. Also, detailed microbiological, physicochemical, sensory and other investigations have been conducted in this area [4,5].

According to Serbian Regulation on the quality of ground meat, meat preparations and meat products [6], fermented sausage can legally contain category 1 or 2 domestic pork, beef or equine meat, category 1 poultry meat and game meat, solid fat tissue, and additives, mixed, and which, after filling in casings, are conserved by drying and fermentation, with or without smoking. Additives for fermented sausages can be salt, curing salt, spices, spice extracts, sugars, additives, starter culture and beverages (wine and others). Fermented sausages are produced as fermented dry sausages, fermented semi-dry sausages for cutting and fermented sausages for spreading. Fermented dry sausages must contain less than 35% water. The drying process is carried out at a low temperature, and only then does sausage get its characteristic, spicy aroma, solid consistency and extended shelf life of the product during the ripening process [7]. Fermented dry sausages vary greatly, and their diversity depends on the country/region, climate, heritage, and culture [8,9,10,11]. There are many formulations for sausage batters, even for products with the same name. The time, temperature and moisture during



the drying process are variable parameters which influence the quality of ready to eat the product [12,13].

The retail market hosts different types of fermented sausages such as kulen, winter salami, srem sausage, sudžuk and tea sausage, but other types of related products also exist. The name *tea sausage* originates from the German word *tee wurst*, which refers to sausage that was produced in the town of Rugenwald in the 19<sup>th</sup> century, nowadays called Darlowo and situated in Poland. This type of sausage was prepared from pork meat and solid fat tissue, and after stuffing into pork small intestine and a quick and short ripening period, it was cut and served in sandwiches with tea, thus the name *tee wurst*.

In the former Yugoslavia, production of tea sausage started just over half a century ago in the *Mesopromet* meat company in Zemun. This type of sausage is mostly industrially produced, which means that the quality of this product is not standardised, but it is acceptable for the majority of the population because it is characterised by an attractive outward appearance, good grinding ability and pleasant aroma. Nowadays, the national market offers dry fermented sausages with similar sensory properties, but, unfortunately, with an overemphasized acidic flavour, often unacceptable to consumers [14,15].

The aim of this paper is to point out the existence of differences in the sensory and chemical parameters of tea sausages produced using two different recipes. These recipes differ only according to the quality of raw pork meat.

## 2. Materials and Methods

Tea sausage was produced according to two recipes, which differed only in the category of some meat ingredients used, while the technological production process was the same for both sausage groups. The tea sausage-group 1 recipe included slightly better quality raw pork meat. Table 1 shows the percentage distribution of the ingredients used.

**Table 1.** The ingredients used to produce tea sausages

Tea sausage-group 1		Tea sausage-group 2	
Raw material	Percentage (%)	Raw material	Percentage (%)
Pork meat category 1	29	Pork meat category 1	25
Pork meat category 2	24	Pork meat category 2	28
Beef meat category 1	19.1	Beef meat category 1	19.1
Solid fat tissue	25	Solid fat tissue	25
Nitrite salt	2.5	Nitrite salt	2.5
RADA <sub>ferm</sub>	0.05	RADA <sub>ferm</sub>	0.05
Dextrose	0.2	Dextrose	0.2
Ascorbic acid	0.05	Ascorbic acid	0.05

In this trial, the technological production processing was in industrial conditions. The raw materials, pork and beef meat (3°C), solid fat tissue (-5°C) were minced in the cutter. After that, other ingredients were added, while starter culture was added at the end of the process. The homogenization was carried out until a 3 mm granulation mosaic was obtained. Tea sausage batters were stuffed using a vacuum filler into collagen casings. Sausages were then hung on horizontal bars of drying racks and left in the anteroom of the automatic air conditioning chamber for about 4 h. This procedure is carried out in order to optimize the process of fermentation/ripening, as the temperature of the filling needs to be raised as near as possible to the optimal temperature (recommendation: to achieve at least 18-19°C, and ideally to 22-24°C) before the fermentation process starts to ensure optimal conditions for the metabolism of starter cultures. The production process (fermentation/drying and smoking, ripening) was a combination of automatic air conditioning chamber and traditional smoke chamber. This process lasted for 23 days.

### 2.1. Laboratory analyses

After production, the sausages were analysed in sensory and chemical laboratories accredited according to SRPS ISO/IEC 17025:2006.

#### 2.1.1. Sensory analyses

Sensory properties of sausages (appearance, surface colour, cross-section colour, cross-section, odour, taste, consistency, salinity, seasoning, overall acceptability) were assessed using a quantitative-descriptive test [17], with a grading scale from one to five (1 = unacceptable, 5 = extremely acceptable) (Table 2). A five-person panel was assembled in order to evaluate the sensory properties. Panellists were previously tested for detection and recognition of various tastes (SRPS 3972, 2001) [18] and odours (SRPS 5496, 2002) [19]. Sensory property results were the median value given by the five panellists (Figure 1).

**Table 2.** Numerical descriptive scale for the assessment of sensory properties

Number rating	Descriptive rating
5	extremely acceptable
4	very acceptable
3	acceptable
2	at the margin of acceptability
1	unacceptable

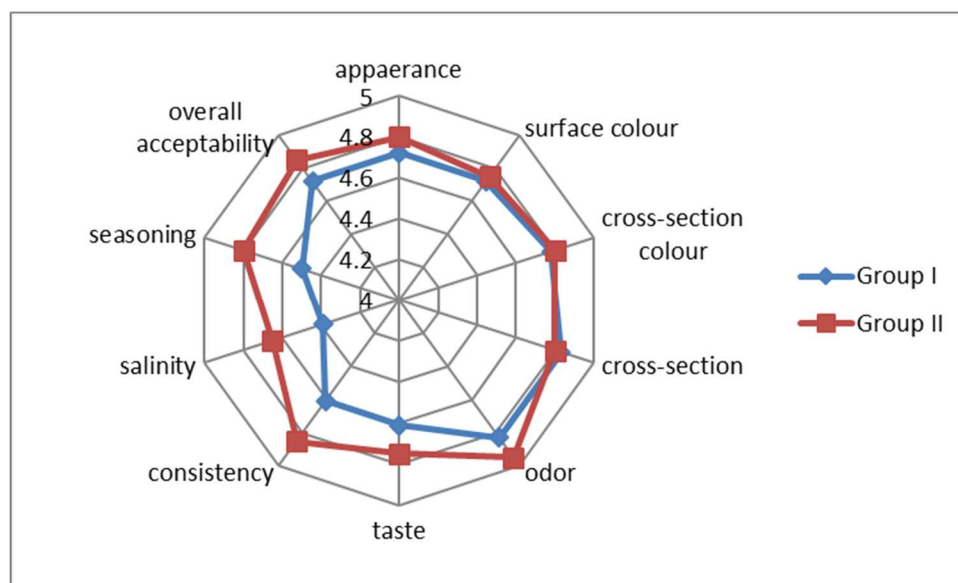
#### 2.1.2. Chemical analyses

After sensory evaluation, samples from each tea sausage were taken for chemical composition analysis. Total fat content [20], NaCl [21], hydroxyproline content [22], moisture content [23] and pH [24] were determined using standard references methods. Nitrogen content was determined by an in-house method, the Kjeldahl method and protein estimated by multiplying the nitrogen content by 6.25 (Kjeltec Auto 1030 Analyzer, Tecator, Sweden), while the sodium chloride was determined by AOAC 24.010 method.

## 3. Results and Discussion

### 3.1. Sensory Properties

Two variants of tea sausage with different categories of pork meat were prepared, while the amount of beef meat, solid fat tissue and other ingredients were the same. The results of sensory analyses by professionally trained assessors are presented in Figure 1.



**Figure 1.** Sensory properties of tea sausage (group I and group II)

The obtained results showed the average scores for all tested sensory properties were similar between the two tea sausage groups. A higher rating was given to tea sausage-group 2, which contained a slightly higher amount category 2 pork meat. These higher scores reflected the better odour, taste and consistency of tea sausage-group 2, while the cross-section was slightly better in tea sausage-group 1, which had a higher amount of category I pork meat. The odour and taste, as well as the other sensory properties of fermented products were influenced by the quality of raw material, ingredients, the metabolic activity of the microbiota present, the physicochemical changes due the drying and ripening processes, and enzymatic degradation of proteins and fats [25,26]. Sausages with a smaller content of fatty tissue are less juicy, have a more solid consistency, and the surface is uneven and wrinkled [27].

### 3.2. Chemical characteristics

The chemical composition of the two different groups of tea sausages is shown in Table 3. Results show that tea sausage-group 1 has higher protein content (27.95%) than tea sausage-group 2, which had 25.25% protein in meat, while the content of collagen in meat protein was similar in both sausage groups. The final protein contents in these sausages were similar to the majority of reported protein contents in a range of different fermented sausages [28, 29]. Moisture contents in our sausages were less than 35%. Low moisture content is typical of similar products from Greece, Hungary, and Croatia [30], and is a consequence of not only drying, but also of the quality and quantity of the input ingredients for the raw sausage, which resulted in relatively high contents of fat (37.00-40.85%) and protein (25.25-27.96%) in the final products. The ingredients used in the two sausage groups did not influence the salt content in finished sausages.

The naturally fermented dry sausages from the Mediterranean region are generally characterized by low acidity with a final pH ranging from 5.2 to 6.4 [28,31], which concurs with our results. According to Heinze and Hautzineru [32], the water activity ( $a_w$ ) of fermented dry sausage is in the range from 0.70 to 0.96, but mostly is 0.91. in our study,  $a_w$  was 0.832 and 0.827 for group 1 and 2 tea sausages, respectively.



**Table 3.** Chemical composition of tea sausages

Traits	Tea sausage-group 1	Tea sausage-group 2
Protein (%)	27.95	25.25
Collagen (%)	6.92	7.01
Water (%)	28.84	27.16
Fat (%)	37.00	40.85
Salt (%)	3.92	3.85
pH value	5.26	5.25
a <sub>w</sub> value	0.832	0.827

#### 4. Conclusion

The results of this study demonstrated that different quantity and quality of pork meat did not have a great influence on the sensory properties of tea sausage. However, these differences in quantity and meat quality resulted in different protein and fat contents in the final products. Therefore, the raw materials and the other ingredients used in the technological production process had an influence on product quality.

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# Effect of cholesterol-lowering starter cultures in smoked sausages on the formation of bioactive peptides and lipid profile in triton-induced hyperlipidemic rats

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**Abstract.** Three types of sausages were studied: without starter cultures; with experimental starter cultures from Moscow State University of Food Production collection; with starter culture Bactoform SM 194 (Chr. Hansen). No pathogenic bacteria in any sample were revealed. According to the results of PCR-RT, the number of lactic acid bacteria in all samples was about the same -  $1 \times 10^9$ - $2 \times 10^9$  CFU/g. T-RFLP analysis shows the maximum number of lactobacilli sausages with experimental starter cultures averaged 69.59% of the total microbiota. The study of the protein profile of raw smoked sausages showed changes in protein fractions and presumably formation of biologically active peptides. A wide range of peptide mass peaks, with certain differences, was obtained by mass spectrometry. Feces of rats (groups 1-5) were studied by T-RFLP. The proportion of lactobacilli was 2.09%, 2.65% and 2.35%, in groups 3-5 respectively. The serum atherogenic index did not differ significantly between the groups due to the low content of non-LDL and non-HDL cholesterol in control rats compared to the other groups. The greatest decrease of serum cholesterol concentration was measured in rats that consumed sausages with experimental starter cultures, mainly due to almost 3-fold ( $P < 0.05$ ) decrease in cholesterol of low-density lipoproteins compared with the control.

## 1. Introduction

The proteome of meat and its by-products is a good source of not only essential nutrients, but also bioactive sequences inhibiting angiotensin-converting enzyme (ACE), or with antioxidant, opioid, immunomodulatory, prebiotic, mineral-binding, cholesterol-lowering and antimicrobial activity [1-7]. The possibility of forming various bioactive peptides through microbial proteolysis has been studied in recent years [8,9]. Thus, some lactic acid bacteria, such as *Lactococcus lactis* and *Lactobacillus helveticus*, generate bioactive peptides during fermentation. This system consists of a number of different intracellular peptidases, including endopeptidases, aminopeptidases, dipeptidases, and tripeptidases [10-11]. However, proteolytic enzymes released by lactic acid bacteria are very different according to the species and strains, and therefore, generated bioactive peptides from these bacteria can belong to different groups [12,13].



Sanz et al. [14] evaluated the activity of proteases and aminopeptidases of *Lactobacillus casei* CRL 705 on muscle proteins. Proteinase activity of whole cells caused degradation of a large number of sarcoplasmic proteins; partial hydrolysis was also associated with cell extracts. Peptide profiles were strongly changed, and more significant generation of free amino acids was noted, when whole cells were combined with cell extracts. Basso et al. [15] compared the proteolytic activities of *Lactobacillus sakei* DSM 6333, *Lactobacillus plantarum* B21 and *Lactobacillus farciminis* DSM 201 84 on meat sarcoplasmic proteins. All strains demonstrated proteolytic activity, especially against muscle glycogenphosphorylase isoform and glyceraldehyde-3-phosphate dehydrogenase.

These studies presented data on the individual effects of starter cultures on the proteolytic changes of meat product raw materials. Of course, the hydrolytic effect of starter cultures, individually or in combination, on the protein profile of meat products is obvious. In our current work, we evaluate the effect of starter culture compositions on the supposed formation of biologically active peptides during fermented sausages processing. It was also previously found that combined starter cultures reduced cholesterol more extensively than single strains [16,17]. The influence of fermented sausages on the fecal microflora and lipid profile of rats with a model of Triton-induced hyperlipidemia was evaluated.

## 2. Materials and Methods

Raw smoked sausages contained beef, horse meat, beef fat, soy protein and spices and were processed by Ekoprod (Ivanteevka, Moscow region). The technology consisted of the following stages: freezing of meat raw materials at -1 to -3 °C, homogenization in a cutter, filling the cases with minced meat, fermentation at 3°C for 5-7 days, smoking at 13-15 °C for 2-3 days and drying for 5-7 days at 15 °C. Smoking and drying processes were repeated two or three times until the product ready. Three types of sausages were processed: BSK – without starter cultures; SKK – with starter cultures from Moscow State University of Food Production (MSUFP) collection (*Lactobacillus sakei* 104 (B-8906), *Pediococcus pentosaceus* 28 (B-8888) and *Staphylococcus carnosus* 108 (B-8953)); SKXX – with starter culture Bactoform SM 194 Chr. Hansen (*Pediococcus pentosaceus*, *Staphylococcus carnosus*, *Staphylococcus xylosus*, *Lactobacillus sakei* and *Debaryomyces hansenii*).

T-RFLP was used to analyze the microbiota of raw smoked sausages. PCR amplification of 16S bacterial rRNA genes was performed using 63F primers labeled at the 5'-end (D4-WellRed fluorophore) and 1492R (Bige LLC, Russia). Amplified fragments were isolated on agarose gel, and then restriction of DNA amplicons from the reaction mixture was conducted. Samples were precipitated, dissolved in SLS Sample Loading Solution (BeckmanCoulter, USA) with the addition of 600 BP molecular weight marker (BeckmanCoulter, USA) and separated by capillary electrophoresis with fluorescence detection using an automatic sequencer CEQ8000 (BeckmanCoulter, USA). Calculation of peak sizes and areas was performed using the FragmentAnalysis software (BeckmanCoulter, USA). Identification of T-RFLP-weights was carried out using FragmentSorter software (University of Idaho, USA).

The total number of microorganisms and lactobacilli in sausages was determined by PCR-RT, using a set of reagents for PCR-RT with Taq DNA-polymerase and enzyme activity inhibiting antibodies in the presence of the dye EVAGreen (LLC NPO DNA-Technology, Russia) according to the manufacturer's recommendations.

Two-dimensional electrophoresis (2DE) was performed according to the method of O'Farrell with isoelectric focusing in ampholine pH gradient (IEF-PAGE). Subsequent detection of the proteins was carried out by staining with silver nitrate (Panreac, Spain). Protein fractions were excised from the gel, ground and submitted to trypsinolysis (Sigma, Germany). The peptides obtained were investigated by MALDI-TOF MS and MS/MS mass spectrometry on an Ultraflex MALDI-TOF mass spectrometer (Bruker, Germany) with UV laser (336 nm) in the positive ion mode and in the molecular weight range of 500-8000 Da with calibration according to known peaks of trypsin autolysis. Mass spectra of tryptic peptides were analyzed using the Mascot program.

T-RFLP-analysis of animal fecal microbiota included the following stages: separation of the total DNA of microorganisms; PCR amplification of bacteria gene fragments (16S rDNA) with

fluorescence primers (usually the 5' end) and 63F CAGGCCTAACACATGCAAGTC and 1087R TACGGHTACCTTGTTACGACTT (Bige LLC, Russia); enzymatic treatment of amplificate using endonucleases HaeIII, HhaI and MspI according to recommendations of the manufacturer (Fermentas, Lithuania) (usually the restriction enzyme that recognizes a sequence of four nucleotides was used); separation of restricted DNA fragments in a polyacrylamide gel in a sequencer CEQ 8000 (Beckman Coulter, USA) together with a fluorescent DNA marker of known size, Standart-600 (Beckman Coulter, USA). The sequencer was equipped with a computer program for automatic calculation of fragment length – Fragment Analysis (Beckman Coulter, USA), based on the comparison of electrophoretic mobility of fragments of each sample with the sample length standards. Each peak in T-RFLP-grams corresponded to one type of microorganism, while the fluorescence intensity of the peak describes its percentage in the microbial community. Determination of the phylogenetic origin of the microorganisms was performed using the programs and databases FragSort (University of Idaho, CIIIA) <http://mica.ibest.uidaho.edu/trflp.php>.

The study of cholesterol-lowering effects of raw smoked sausages was carried out on Triton-induced hyperlipidemic rats. Mature Wistar male rats (220±5 g) were formed in statistical groups ( $n_{i=1-5}=10$ ) by randomization according to body weight. Previously, during 20 days, group 3 animals were administered BSK, group 4 – SKXX, and group 5 – SKK. All sausages were mixed with standard chow. Group 1 consisted of intact rats ( $n=10$ ), group 2 of control animals kept under equivalent conditions. On day 21, Triton X-100 (previously dissolved in a physiological solution) was injected intraperitoneally at a dose of 300 mg/kg of weight to rats in groups 2-5 ( $n=40$ ), while group 1 ( $n=10$ ) animals were injected intraperitoneally with an equivalent volume of sterile water. On day 22, the animals were euthanized (VETtech, UK), and blood samples for biochemical studies were taken.

Biochemical investigations were carried out on an automatic analyzer BioChem FC-360 (HTI, USA) according to instructions applied to measurement kits (HTI, USA). Total cholesterol (TCL), triglyceride (TG), cholesterol low-density lipoproteins (CL LDL) and cholesterol high-density lipoproteins (CL HDL) levels were measured in rat sera. Atherogenic index was calculated by the following formula:  $(AI) = (TCL - CL\ HDL) / CL\ HDL$ .

STATISTICA 10.0 software was used in this study for the statistical analyses. The results were calculated as mean ± standard error ( $M \pm SE$ ). Significant differences were tested by one-way ANOVA, followed by Tukey's test. Differences with P-values less than 0.05 were considered as statistically significant.

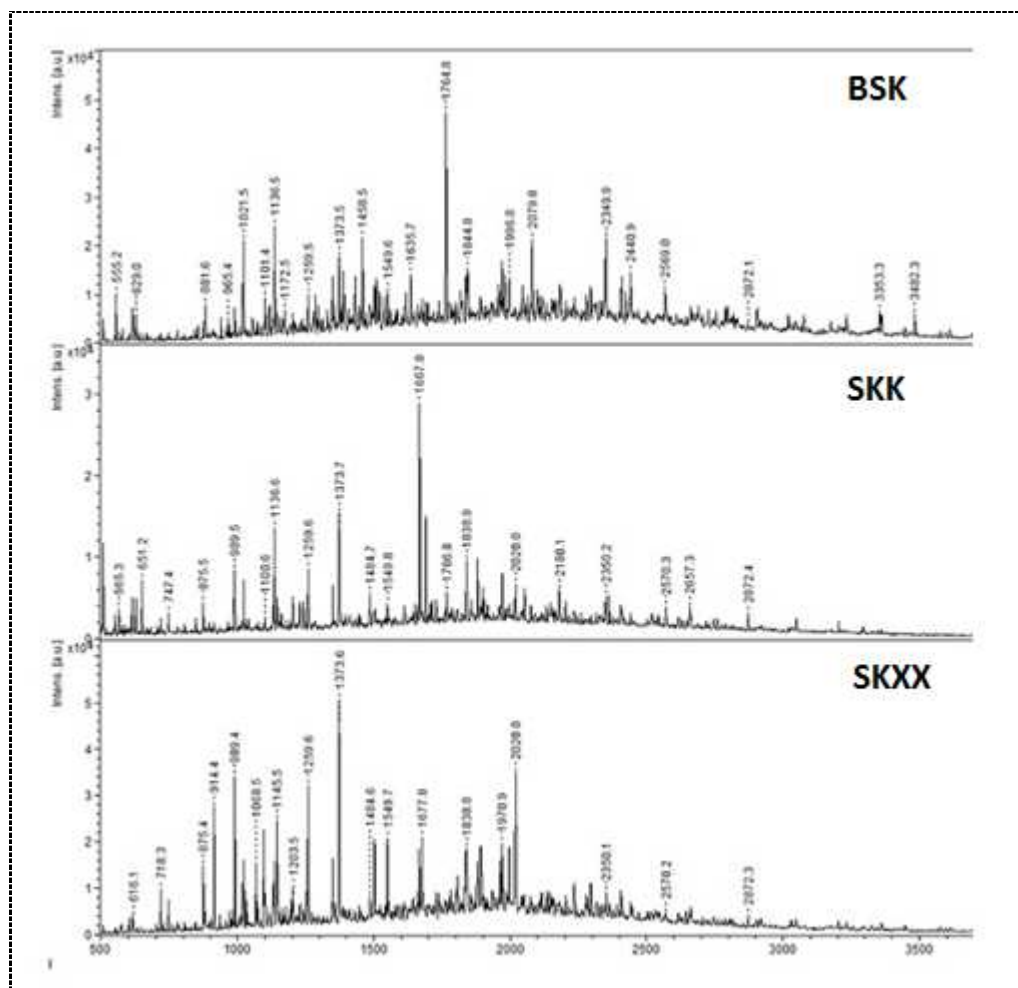
### 3. Results and Discussion

Pathogenic microorganisms were not detected in any of the experimental sausages. According to results of T-RFLP, the maximum number of lactobacilli occurred in the SKK sausage and averaged 69.59% of the total microbial population, in BSK – 66.40%, in SKXX – 57.90%; coefficient of variation was less than 5%. According to PCR-RT, the number of lactic acid microorganisms in all sausages was almost the same,  $1 \times 10^9$  CFU/g in BSK and SKK, and  $2 \times 10^9$  CFU/g in SKXX. The large population of lactic acid microorganisms in BSK detected by T-RFLP and PCR-RT could be explained by development of spontaneous lactic microbiota, which was not necessarily homofermentative and could negatively influence the quality of fermented meat products. Staphylococci were also found, subsequently identified to strain level. Thus, the microbiota of all raw smoked sausages was non-pathogenic, mostly lactic acid bacteria and uncultivated bacteria.

2DE of sausages showed that starter cultures from the MSUFP collection retarded the formation of N- and C- terminal fragments of myosin heavy and light chains; actomyosin complex decomposed more slowly compared with commercial starter culture, but a number of enzymes, such as muscle creatin phosphokinase and enolase, were degraded faster by aldolase A. Electrophoregrams of BSK sausage confirmed the degradation of protein fractions.

A wide range of peptide mass peaks in the studied sausages was obtained by mass spectrometry, but there were some differences between treatments. BSK sausage was characterized by the widest mass spectrum ( $m/z$  500-5000) and a large number of mass peaks (23 peaks). The number of mass peaks

reduced to 20 in SKK and was mainly in the range of  $m/z$  500-3500. The spread of mass peaks was decreased, especially in peaks with  $m/z > 2500$ . The total number of peaks reduced to 19 in SKXX, and a portion of the peaks moved to  $m/z$  800-1800. Specific biologically active peptides should be identified during further studies (Figure 1).



**Figure 1.** Mass spectra of raw smoked sausage peptides

The effect of the diet containing raw smoked sausages with starter cultures on bacterial fecal community composition of experimental animals was studied by T-RFLP analysis (Table 1).

**Table 1.** The content of microorganisms in the faeces of experimental animals, %

Microorganisms	Group 1	Group 2	Group 3	Group 4	Group 5
Normal fecal microorganisms					
Cellulolytic bacteria, including:					
<i>Bacteroidetes</i> spp.	42.65±1.35	54.12±1.66	39.10±1.21	68.39±2.10	35.33±0.98
<i>Clostridiaceae</i> spp.	0.75±0.54	7.43±0.74	5.62±0.03	5.72±0.10	2.27±0.49
<i>Ruminococcaceae</i> spp.	0.44±0.22	7.52±0.39	11.14±0.74	21.63±1.04	10.88±0.92
<i>Eubacteriaceae</i> spp.	16.01±0.90	18.56±0.85	11.18±0.86	12.77±0.78	8.11±0.81
	20.99±1.10	18.75±0.91	8.00±0.39	24.82±1.27	11.60±0.87

<i>Lachnospiraceae</i> spp.	4.46±0.66	1.86±0.15	3.16±0.24	3.45±0.17	2.47±0.42
<i>Lactobacillales</i> spp.	0.74±0.17	1.88±0.17	2.09±0.21	2.65±0.15	2.35±0.31
<i>Bifidobacteriales</i> spp.	0.00±0.10	0.09±0.04	0.27±0.07	0.39±0.04	4.23±0.56
<i>Bacillales</i> spp.	2.65±0.68	3.27±0.22	2.77±0.35	2.09±0.53	5.72±0.67
<i>Selenomonadales</i> spp.	21.75±1.08	7.36±0.60	18.50±0.97	1.33±0.24	10.38±0.84
Potentially pathogenic microorganisms					
<i>Enterobacteriaceae</i> spp.	0.13±0.05	0.42±0.08	0.21±0.08	1.62±0.17	0.64±0.09
<i>Actinobacteria</i> spp.	3.18±0.65	3.70±0.15	6.70±0.88	3.65±0.55	4.73±0.61
<i>Staphylococcus</i>					
<i>Staphylococcus</i> spp.	0.22±0.10	0.13±0.07	0.37±0.11	0.11±0.02	0.46±0.07
Pathogenic microorganisms					
<i>Fusobacteria</i> spp.	6.23±0.22	4.85±0.32	3.15±0.56	2.69±0.41	2.04±0.24
<i>Peptococcaceae</i> spp.	2.33±0.17	2.92±0.17	0.35±0.12	2.50±0.26	0.39±0.11
<i>Pasteurellaceae</i> spp.	0.00	0.97±0.07	0.00	0.00	0.00
<i>Campylobacteriaceae</i> spp.	0.00	0.13±0.10	0.10±0.07	0.00	0.00
Transitory and uncultivated microorganisms					
<i>Pseudomonadaceae</i> spp.	0.93±0.59	2.58±0.10	0.05±0.14	2.20±0.60	0.17±0.10
Uncultivated microflora	19.19±0.94	17.58±0.75	26.34±1.10	12.38±0.55	33.56±1.32

The percentages of cellulolytic bacteria, lactobacilli and *Selenomonadales* spp. in the animal feces were high. The percentage of lactobacilli in groups 3-5 was the highest and amounted to 2.09%, 2.65% and 2.35% of the total population, respectively, perhaps because diet of animals in these groups included raw smoked sausage with lactic acid bacteria. In feces of group 1 experimental animals, bifidobacteria were not detected. The greatest population of bacilli (5.72%) was found in the feces of group 5 rats. Staphylococci were found in all groups. The proportion of *Selenomonadales* spp. in all feces was high, except in feces of group 4 rats (1.33%). The content of actinobacteria and enterobacteria in all feces was approximately the same, except feces of group 3 rats, in which the percentage of actinobacteria was 6.70%.

The presence of pathogenic microorganisms was observed in the rat feces: *Campylobacter* was not detected in feces of animals from groups 1, 4 and 5; peptococci were found in small quantities in all feces. The fusobacteria content was slightly increased in groups 1 and 2 compared with the other rat groups. *Pasteurella* was found only in rat faeces from group 2 and amounted to 0.97% of the fecal population. Among transitory microflora associated with food, *Pseudomonas* were identified. Uncultivated bacteria were observed in all feces, the percentages of which ranged from 12.38% to 33.56%.

The analysis of changes in serum lipid profile of experimental animals 24 h after Triton X-100 injection revealed that in control animals (group 2), the content of TCL significantly increased by 70.0% ( $P<0.05$ ) compared with intact rats (group 1), mainly due to an increase in CL LDL by 3.9-fold ( $P<0.05$ ), but HDL CL also increased by 46.6% ( $P<0.05$ ), and the concentration of TG did not change statistically significantly in the experimental groups. The greatest decrease of TCL was observed in group 5 (SKK) and amounted to 32.0% ( $P<0.05$ ) compared with the control (group 2), mainly due to a decrease in CL LDL by almost 3-fold ( $P<0.05$ ). However, serum AI of animals that consumed experimental sausages did not differ significantly from the control (group 2) due to the low content of CL non-LDL and non-HDL in control rats, which was quite high compared with the other groups (Table 2). Thus, it was shown that sausages with starter cultures from the MSUFP collection demonstrated a cholesterol-lowering effect.

**Table 2.** Serum lipid profiles of experimental animals

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
TCL, mmol/L	2.13±0.03 <sup>#</sup>	3.62±0.15*	2.78±0.13* <sup>#</sup>	2.48±0.11 <sup>#</sup>	2.46±0.14 <sup>#</sup>
TG, mmol/L	0.53±0.03 <sup>#</sup>	0.47±0.03	0.56±0.05	0.49±0.04	0.55±0.05
CL LDL, mmol/L	0.53±0.01 <sup>#</sup>	2.05±0.08*	0.85±0.04* <sup>#</sup>	0.79±0.03* <sup>#</sup>	0.69±0.05* <sup>#</sup>
CL HDL, mmol/L	1.03±0.01 <sup>#</sup>	1.51±0.06	1.09±0.07 <sup>#</sup>	0.99±0.03 <sup>#</sup>	1.04±0.05 <sup>#</sup>
CL non-LDL and non-HDL	0.60±0.02 <sup>#</sup>	0.07±0.01*	0.84±0.08* <sup>#</sup>	0.71±0.07 <sup>#</sup>	0.72±0.05 <sup>#</sup>
AI	1.10±0.03	1.41±0.06*	1.57±0.08*	1.51±0.07*	1.35±0.05*

\* – significant difference compared with group 1 (P<0.05), <sup>#</sup> – significant difference compared with group 2.

Gallego et al. [18] described proteolysis of three traditional European dry fermented sausages from Spain, Italy and Belgium, as well as peptide and free amino acid profiles. Their obtained data was explained by differences in composition, conditions of processing and starter culture used in each type of sausage. Moreover, the combined action of muscle and microbial enzymes in these products contributed to the formation of bioactive peptides, demonstrating ACE-inhibitory properties and antioxidant activity. Spanish and Belgian fermented sausages showed the maximum values of ACE inhibition activity, while the Belgian sample showed the highest antioxidant inhibitory activity against the 2,2-diphenyl-1-picrylhydrazyl radical.

Elevated cholesterol level in serum is associated with risk of cardiovascular diseases. Cholesterol increases lipid peroxidation, protein oxidation and the production of free radicals, impairs the antioxidant system (SOD, CAT, GPx and GSH), as well as the activity of ATPase and causes histopathological disorders. Bioactive peptides can be considered as a tool for the prevention or treatment of these disorders. Peptides, identified as cholesterol-lowering, include lactostatin (IIAEK), enterostatin (VPDPR), peptides DPR, LPYP and LPLPR [19]. Peptides of this type are expected to demonstrate different mechanisms of action and can increase the excretion of bile acids with feces, can bond with phospholipids, and can display cholesterol-lowering activity.

Currently, bioactive peptides with proven cholesterol-lowering activity are obtained from various sources [19-21]. Thus, *L. helveticus* KII13, isolated from fermented cow's milk and producing bioactive tripeptides isoleucyn-proline-proline and valine-proline-proline, significantly reduced serum cholesterol and LDL levels in mice with induced atherogenic hypercholesterolemia [20]. Similarly, administration of *L. plantarum* 14 *per os* to mice C57/BL6 that were fed high fat diet led to decrease in the mass of adipose tissue, serum cholesterol and leptin; significant changes in weight gain and concentrations of conjugated linoleic acid in serum were not observed [21].

In a number of studies, it was noted that the addition of protein hydrolysates and biologically active peptides of animal origin to the diet of rats with high cholesterol can play a role in reducing atherogenic parameters [22-24]. Dietary additives with kimchi powder or *L. plantarum* in fermented sausages are effective in reducing the level of lipids, cholesterol and atherogenic index in rat serum. The concentrations of triglycerides in serum of experimental animals that consumed fermented sausage with kimchi and *L. plantarum* were not significantly different compared with the control group. The levels of total cholesterol, low-density lipoproteins and high-density lipoproteins in the serum of rats that consumed sausage with kimchi were significantly lower than in the control. The level of free cholesterol in serum and atherogenic index of rats that consumed sausage with kimchi and *L. plantarum* were significantly lower than in the control [22]. Drotningvik et al. [23] studied the influence of marine fish protein hydrolysates containing peptides on typical markers of metabolic disorders in the model of obesity and diabetes of fa/fa Zucker rats [23]. It was shown that diets containing hydrolyzed herring or salmon proteins can affect growth, lipid metabolism, regulation of glucose levels after meals and the composition of fatty (mono- and polyunsaturated) acids in serum



and adipose tissue in rats with a model of metabolic syndrome. Protein hydrolysates of zebra blenny, containing biologically active peptides with antioxidant activity, recover disorders caused by the biochemical histopathological effect of cholesterol in rats with hypercholesterolemia [24].

#### 4. Conclusions

The results obtained expand the knowledge of proteolysis, which occurs during processing of sausages fermented with starter cultures. Their potential as natural sources of biologically active peptides is evident and gives additional value to these products, which are still not well enough studied. Specific peptide sequences responsible for the observed biological activities should be identified during further research. Consumption of raw smoked sausages with cholesterol-lowering starter cultures by experimental animals led to reduction of serum cholesterol concentration compared with control. The serum atherogenic index of animals that consumed raw smoked sausage with starter cultures from the MSUFP collection was the lowest. At the same time, the atherogenic index in experimental groups that consumed SKXX and BSK sausages tended to increase, while there was a decrease of atherogenic index in serum of animals that consumed SKK, although the differences were not statistically different in comparison with the control. Therefore, there is an obvious need for further research in this area.

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## Element concentration and fatty acid composition of Serbian bee bread

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**Abstract.** The element concentration (Cu, Fe, Zn, Mn, Cr, Co, Ni, Se, K, Na, Ca and Mg), heavy metal concentration (Cd, Hg, Pb and As) and fatty acid composition of 12 Serbian bee bread samples from different geographical origins were examined. The element concentration was examined using ICP-MS, and total lipids for fatty acid determination were extracted from homogenized bee bread samples with hexane/isopropanol mixture by accelerated solvent extraction. Potassium was the major element, ranging between 5515±361.20 mg/kg and 7487±381.50 mg/kg. The highest As and Pb concentrations were found in bee breads from Lazarevac. This bee bread also contained the highest level of PUFA and SFA. Also, the n-6/n-3 ratio ranged between 0.86±0.28 and 1.40±0.05, indicating bee bread can be a good source of unsaturated fatty acids. Bee bread could be useful in monitoring environmental contamination by heavy metals (Cd, Hg, Pb and As), although complex studies of all bee products give sufficient information on this topic.

### 1. Introduction

Honey bee products, due to their nutritional and medical properties, are widely used in human diet and medicine. Therefore, many studies have presented the chemical composition of these products. A majority of publications refer to honey, wax, or propolis composition [1-7]. However, the composition of bee bread, including fatty acid composition and element concentration, has not been equally studied. Fermented bee pollen is called bee bread (Figure 1) and mainly includes pollen, honey, and secretions of bees' salivary glands [8]. This work presents, for the first time, the fatty acid composition and element concentration of 12 samples of beebread, obtained from different geographical locations in Serbia, where bee bread, due to its nutritional and physiological properties, is used in human diets.





**Figure 1.** Bee bread (in beekeeping frame) (J. Ciric)

## 2. Material and Methods

A total of 12 bee bread samples were obtained from apiaries located in different Serbian regions (I – Gornji Milanovac, II – Lazarevac and III – Ležimir) between May and August of 2018. The bee bread samples were hand collected from four healthy beehives in apiaries with 30-70 colonies and kept at -20 °C.

Approximately 0.5 g of homogenized bee bread was transferred into a Teflon vessel with 5 ml nitric acid (67% Trace Metal Grade, Fisher Scientific, Loughborough, UK) and 1.5 ml hydrogen peroxide (30% analytical grade, Sigma-Aldrich, St. Louis, MO, USA) for microwave digestion. The microwave (Start D, Milestone, Sorisole, Italy) program consisted of three steps: 5 min from RT to 180 °C, 10 min hold at 180 °C, and 20 min cooling. Analysis of the following 16 elements: Fe, Zn, Cu, Mn, Se, Cr, Co, Ni, Na, K, Mg, Ca, Cd, Pb, Hg and As, was performed by inductively coupled plasma mass spectrometry (ICP-MS) (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany). Multielemental internal standard was introduced into the ICP-MS during the measurements.

Total lipids for fatty acid determination were extracted from homogenized bee bread with hexane/isopropanol mixture by accelerated solvent extraction (ASE 200, Dionex, GmbH, Idstein, Germany). After evaporation of solvent until dryness under a stream of nitrogen, total lipids were converted to fatty acid methyl esters (FAME) by trimethylsulfonium hydroxide. FAMES were determined using a Shimadzu 2010 gas chromatograph equipped with flame ionization detector (FID) and cyanopropyl HP-88 capillary column (100 m x 0.25 mm x 0.20 mm) [9]. Temperature of the injector and detector were 250 °C and 280 °C, respectively. FAMES were identified on the basis of relative retention time, compared with the relative retention times of the individual compounds in a standard mixture of fatty acid methyl esters, Supelco Component 37 FAME mix (Supelco, Bellefonte, USA). Quantification of fatty acids was determined relative to an internal standard, heneicosanoic acid, C21:0. The level of fatty acids is expressed as a percentage (%) of the total identified fatty acids.

The statistical analysis was performed using the GraphPad Prism version 7.00 software. The fatty acid composition and concentrations of elements in bee bread samples were expressed as the mean  $\pm$  standard deviation and were subjected to analysis of variance (One-way ANOVA). The parameters were analyzed using the Student's t-test at the probability of 0.01.

## 3. Results and Discussion

The element concentrations (Cu, Fe, Zn, Mn, Cr, Co, Ni, Se, K, Na, Ca and Mg) and heavy metal concentrations (Cd, Hg, Pb and As) of the bee bread are shown in Table 1. The Na content differed widely among the geographical locations. Bee bread from Ležimir was characterized by the significantly highest mean Na concentration ( $p < 0.01$ ). The highest Mg and K concentrations were

found in the bee bread from geographical location I (Gornji Milanovac). Potassium was the major element determined in the bee breads. The mean Ca content in the bee bread ranged between  $1190 \pm 76.38$  mg/kg and  $1806 \pm 44.98$  mg/kg. The lowest Mn, Fe and Ni concentrations were measured in bee bread from Gornji Milanovac ( $19.51 \pm 3.74$  mg/kg,  $43.07 \pm 3.84$  mg/kg and  $1.30 \pm 0.26$  µg/kg, respectively). The mean Cr concentration in all tested bee bread was between  $106.90 \pm 12.92$  µg/kg (Ležimir) and  $183.80 \pm 29.77$  µg/kg (Lazarevac). Se was not detected in any of the examined bee bread ( $<0.2$  mg/kg). The concentrations of elements in bee bread have been reported in several studies [10–13], but for Serbian bee bread, data is very limited. However, similar results to ours were reported previously by Stanciu et al. [10], Villaneuva et al. [11], Somerville et al. [12] and Salamanca et al. [13].

The Fe concentration of the bee bread was significantly different ( $p < 0.01$ ) in different locations and ranged between  $43.07 \pm 3.84$  mg/kg (Gornji Milanovac) and  $57.52 \pm 4.28$  mg/kg (Ležimir). Similar results were obtained by Villaneuva et al. [11] and Stanciu et al. [10]. The mean Co concentrations in all tested bee breads was significantly different ( $p < 0.01$ ). The concentrations of Cu and Zn were relatively higher in bee bread collected from Ležimir ( $10.72 \pm 1.67$  µg/kg,  $46.94 \pm 8.37$  mg/kg, respectively).

The concentrations of heavy metals (As, Cd, Pb and Hg) in bee bread collected from apiaries in different locations were presented in Table 1. Data obtained revealed the highest levels of As ( $43.37 \pm 4.39$  µg/kg) and Pb ( $183.20 \pm 8.95$  µg/kg) occurred in bee bread from Lazarevac. On the other hand, the lowest concentrations of Cd ( $32.18 \pm 0.73$  µg/kg) were recorded in bee bread from Gornji Milanovac. Hg was not detected in any of the examined bee breads ( $<1.0$  µg/kg). The differences in concentrations of heavy metals (As, Cd, Pb) in bee bread between the three study locations could be attributable to different local contamination/pollution sources. The main contaminants of bee products are heavy metals [1, 2, 14], and pesticides originating from the environment [15, 16] and from agricultural and apiculture practices [17]. The present study revealed the highest As and Pb concentrations occurred in bee bread from Lazarevac. These results, as previously mentioned, emphasize the likely elevated level of environmental pollution with these heavy metals in this location.

**Table 1.** Element concentrations ( $\bar{X} \pm Sd$ ) in Serbian bee breads from different geographical locations

Element (units)	Geographical location		
	I*	II	III
Na (mg/kg)	$32.90 \pm 1.69^A$	$34.91 \pm 1.50^A$	$49.07 \pm 3.03^B$
Mg (mg/kg)	$845.80 \pm 83.20^A$	$692.20 \pm 39.93^B$	$714.70 \pm 40.64^C$
K (mg/kg)	$7487 \pm 381.50^A$	$5944 \pm 730.40^B$	$5515 \pm 361.20^C$
Ca (mg/kg)	$1190 \pm 76.38^A$	$1266 \pm 248.80^B$	$1806 \pm 44.98^C$
Cr (µg/kg)	$131.30 \pm 15.68^A$	$183.80 \pm 29.77^B$	$106.90 \pm 12.92^C$
Mn (mg/kg)	$19.51 \pm 3.74^A$	$38.15 \pm 4.99^B$	$204.80 \pm 1.17^C$
Se (mg/kg)	$<0.2$	$<0.2$	$<0.2$
Fe (mg/kg)	$43.07 \pm 3.84^A$	$54.97 \pm 6.79^B$	$57.52 \pm 4.28^B$
Co (µg/kg)	$65.50 \pm 9.70^A$	$37.52 \pm 4.65^B$	$55.43 \pm 3.88^C$
Ni (µg/kg)	$1.30 \pm 0.26^A$	$1.33 \pm 0.12^B$	$3.67 \pm 0.30^C$
Cu (µg/kg)	$5.29 \pm 0.25^A$	$4.59 \pm 0.11^A$	$10.72 \pm 1.67^B$
Zn (mg/kg)	$41.59 \pm 3.63^A$	$27.31 \pm 2.25^B$	$46.94 \pm 8.37^A$
As (µg/kg)	$33.27 \pm 9.63^A$	$43.37 \pm 4.39^B$	$20.50 \pm 3.96^C$
Cd (µg/kg)	$32.18 \pm 0.73^A$	$43.50 \pm 4.15^B$	$136.50 \pm 8.48^C$
Pb (µg/kg)	$100.90 \pm 6.06^A$	$183.20 \pm 8.95^B$	$55.85 \pm 6.42^C$
Hg (µg/kg)	$<1.0$	$<1.0$	$<1.0$

\* I – Gornji Milanovac, II – Lazarevac and III – Ležimir; Different letters (<sup>A</sup>, <sup>B</sup>, <sup>C</sup>) show statistically significant different average concentrations between bee breads from different locations,  $p < 0.01$

Table 2 contains data on the fatty acid composition of the investigated bee bread. The average fatty acid compositions of the bee breads from different geographical locations differed significantly ( $p < 0.01$ ). The most abundant fatty acid in bee bread from Gornji Milanovac, Lazarevac and Ležimir was C16:0 ( $39.23 \pm 3.57\%$ ) followed by C18:3n-3. The bee breads contained high levels of saturated (SFA) and polyunsaturated fatty acids (PUFA). The total n-3 fatty acid content was the highest in bee breads from Lazarevac ( $25.64 \pm 2.90\%$ ) and the lowest in bee breads from Ležimir ( $7.30 \pm 0.99\%$ ). The content of n-6 fatty acids varied between  $10.21 \pm 1.97\%$  (Ležimir) and  $22.00 \pm 1.16\%$  (Lazarevac). The most favourable n-6/n-3 fatty acid ratio was found in bee bread from Gornji Milanovac. The proportion of total n-6/n-3, fulfilling the demands of health-conscious consumers (reducing the risk of many diseases), should be from 1 to 5 [18]. Similar results were presented in the studies by Kaplan et al. [19] and Isidorov et al [1].

**Table 2.** Fatty acid composition (% of total fatty acids or fatty acid ratio;  $\bar{X} \pm Sd$ ) of Serbian bee breads from different geographical locations

Fatty acid	Geographical location		
	I*	II	III
C14:0	$0.63 \pm 0.13^A$	$0.41 \pm 0.15^A$	$8.15 \pm 1.09^B$
C15:0	$5.28 \pm 0.04^A$	$5.34 \pm 0.10^A$	$2.98 \pm 0.16^B$
C16:0	$23.00 \pm 2.80^A$	$18.46 \pm 2.60^A$	$39.23 \pm 3.57^B$
C16:1	$0.30 \pm 2.80^A$	$0.32 \pm 1.80^A$	ND <sup>1</sup>
C18:0	$5.54 \pm 0.25^A$	$5.05 \pm 0.66^A$	$4.12 \pm 0.29^A$
C18:1cis	$17.74 \pm 0.03^A$	$17.72 \pm 0.06^A$	$15.82 \pm 0.07^B$
C18:2cis	$20.17 \pm 0.73^A$	$21.00 \pm 1.03^A$	$7.54 \pm 1.02^B$
C20:0	$1.99 \pm 0.04^A$	$1.90 \pm 0.08^A$	$1.33 \pm 0.05^B$
C18:3n-6	ND	ND	$1.06 \pm 0.02$
C18:3n-3	$21.20 \pm 2.79^A$	$24.66 \pm 3.01^A$	$5.37 \pm 0.36^B$
C20:1	$0.92 \pm 0.01$	ND	ND
C20:2n-6	$0.70 \pm 0.04^A$	$0.62 \pm 0.06^A$	$0.70 \pm 0.03^A$
C20:3n-6	$0.39 \pm 0.01^A$	$0.37 \pm 0.02^A$	$1.98 \pm 0.01^B$
C20:3n-3	$0.89 \pm 0.12^A$	$0.97 \pm 0.10^A$	$1.93 \pm 0.01^B$
C24:0	$3.97 \pm 0.48^A$	$3.01 \pm 0.30^A$	$8.51 \pm 0.60^B$
C24:1	$0.15 \pm 0.01^A$	$0.14 \pm 0.01^A$	$1.31 \pm 0.01^B$
SFA	$39.64 \pm 3.75^A$	$34.17 \pm 4.20^A$	$64.30 \pm 5.69^B$
MUFA	$17.86 \pm 0.31^A$	$18.19 \pm 0.29^A$	$17.12 \pm 2.30^A$
PUFA	$42.50 \pm 4.85^A$	$47.64 \pm 3.99^A$	$18.57 \pm 1.58^B$
n-6	$20.97 \pm 1.11^A$	$22.00 \pm 1.16^A$	$10.21 \pm 1.97^B$
n-3	$22.27 \pm 3.69^A$	$25.64 \pm 2.90^A$	$7.30 \pm 0.99^B$
n-3/n-6 ratio	$1.03 \pm 0.10^A$	$1.17 \pm 0.13^A$	$0.71 \pm 0.03^B$
n-6/n-3 ratio	$0.87 \pm 0.37^A$	$0.86 \pm 0.28^A$	$1.40 \pm 0.05^B$

\* I – Gornji Milanovac, II – Lazarevac and III – Ležimir; Different letters (<sup>A, B, C</sup>) show statistically significant differences between bee breads from different locations,  $p < 0.01$ ; <sup>1</sup> ND – Not detected

#### 4. Conclusion

To our knowledge, this study is the first to compare the chemical composition and fatty acid composition of bee breads from different geographical locations in Serbia. The results of the analyses show that beebread contains large quantities of unsaturated fatty acids and sometimes has very favourable n-6/n-3 fatty acid ratios. This composition indicates the high nutritive value of bee bread.

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# Effects of RYR1 gene mutation on the health, welfare, carcass and meat quality in slaughter pigs

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**Abstract.** This study assessed the effects of RYR1 mutation on the health, welfare, and carcass and meat quality in slaughter pigs. Any signs of pneumonia, pleurisy, pericarditis, and liver milk spots were recorded as present or absent. At exsanguination, blood samples were collected and RYR1 genotype, blood lactate and glucose concentrations were determined. The following carcass quality traits were measured: live, hot and cold carcass weights, backfat thickness, loin muscle thickness, lean meat content and skin lesion score. pH and temperature of *M. longissimus dorsi* and *M. semimembranosus* were measured 45 minutes postmortem. Nn pigs were more affected by pneumonia, had higher blood lactate and glucose concentrations and more developed *rigor mortis* than NN pigs. NN pigs had lower daily weight gain, produced lighter carcasses, more fat and less meat than Nn pigs. Meat obtained from Nn pigs was of a lower quality class than meat obtained from NN pigs, as shown by the lower pH and higher temperatures measured 45 minutes *post mortem* in both muscles and higher prevalence of pale, soft and exudative meat. In conclusion, the presence of a mutant n allele in pigs positively affected carcass quality traits, but had a deleterious effect on health, welfare and meat quality.

## 1. Introduction

The major goals of modern pig production have been increased lean meat content and growth rate, which led to a considerable increase in stress susceptibility, decrease in resistance to diseases and impaired meat quality. Besides ordinary selection for higher meatiness, in many pig populations, the improved meatiness is also a result of a high frequency of the ryanodine receptor (RYR1) gene (n), the major gene giving positive effects on meat quantity, but negative effects on pork quality [1-2]. The pigs with RYR1 gene in the form of a recessive homozygote (nn genotype) have been characterized by better feed conversion efficiency, faster growth, superior lean content and conformation compared with pigs free of this mutation (NN genotype), as a result of lower fat and bone proportions and better carcass weight distribution. Nonetheless, nn pigs have also been found to show higher mortality rates during pre-slaughter period, and be more prone to produce pale, soft and exudative (PSE) meat [3-5]. The scientific literature on the characteristics of heterozygous pigs (Nn genotype) is not consistent. Some authors reported that the RYR1 gene in its heterozygous form (Nn genotype) has certain beneficial effects such as a higher lean meat content with little or no effect on pork quality [6]. Other studies indicate the stress carriers (Nn genotype) do have some advantages compared to stress-



negative (NN genotype) pigs, such as better feed efficiency, greater carcass yield and higher meatiness, but at the same time, they do have a higher prevalence of PSE meat [7-9]. Therefore, the aim of this study was to determine the effects of RYR1 mutation on the health, welfare, and carcass and meat quality in slaughter pigs.

## 2. Materials and Methods

### 2.1. Animals and management procedures

The study was conducted in 2017 on 60 slaughter pigs (31 barrows and 29 gilts) with average live weight of approximately 112 kg and 6 months old. All pigs were of the same genetics ([Yorkshire × Landrace] sows sired with Pietrain boars) and originated from the same farm. The farm was a conventional farrow-to-finish herd practicing an all in/all-out management with confined (i.e. indoor) sows, weaners and fattening pigs. The farm has 650 breeding sows and produces about 12,500 fattening pigs a year. Pigs were housed in a finishing facility on a slatted floor, in groups of 20 animals per pen, at an average density of 1.0 m<sup>2</sup> per pig. Animals were fed *ad libitum* with liquid feed during the entire fattening period. The treatment conditions, both before and after slaughter, were identical for all pigs and in accordance with the conventional industrial practice. Pig slaughter and carcass processing were performed at the same small-scale commercial slaughterhouse with a daily slaughter rate of approximately 35 pigs.

### 2.2. RYR1 genotype determination

To identify RYR1 genotype, the blood sample of each pig was collected in a plastic cup from the bleeding wound at exsanguination. The blood was sampled to vacutainers containing K3EDTA anticoagulant. DNA was isolated using the KAPA Express Extract Kit (Kapa Biosystems, Wilmington, Massachusetts, USA) according to the manufacturer's instructions. After extraction of DNA, the RYR1 genotype of the pigs was determined using PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) method. After PCR, DNA fragments with 134 base pairs (bp) were amplified using the KAPA2G Robust HotStart ReadyMix (Kapa Biosystems, Wilmington, Massachusetts, USA) and the following primer sequences [10]: primer 1: 5'-GTG CTG GAT GTC CTG TGT TCC CT-3' and primer 2: 5'-CTG GTG ACA TAG TTG ATG AGG TTT G-3'. The PCR reaction mixture with final reaction volume of 25 µl contained: 1X Master Mix (KAPA2G Robust HotStart ReadyMix, 2X) and 0.2 µM of each of the primers. The amplification reaction was carried out in a FlexCycler (Analytic Jena, Germany) under the following conditions [10]: denaturation at 94°C for 5 min; 35 cycles comprising denaturation DNA at 94°C for 40 s, annealing at 59°C for 40 s, complementary strand polymerization at 72°C for 40 s, and; final extension at 72°C for 5 min. The amplified PCR products were digested with the restriction enzyme FastDigest Hin6I (Fermentas, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C for 5 min according to the manufacturer's instructions. Reaction mixture with final reaction volume 30 µl contained: 10 µl PCR product (~0.1-0.5 µg DNA), 1X buffer and 1 U/µL of the restriction enzyme FastDigest Hin6I. Digestion of this product by FastDigest Hin6I yields two fragments of 84 and 50 bp for stress-resistant (NN genotype), three fragments of 134, 84, and 50 bp for heterozygotes – the stress-carrier (Nn genotype) and only the 134 bp DNA fragment for mutant homozygous – the stress-susceptible (nn genotype) individuals. The digested PCR products were separated by horizontal electrophoresis (Carl ROTH N817.1 minieasy Electrophoresis Unit, Carl Roth, Germany) on 2% agarose gel (NipponGenetics, Tokyo, Japan), stained with ethidium bromide (Sigma-Aldrich Chemie GmbH P.O., Steinheim, Germany). After electrophoresis, gels were analyzed by using a transilluminator (Vilber Lourmat – ETX-20.C 254 nm, Vilber Lourmat, France) and, then, the size of the fragments was compared with a standard of known molecular weight (Gene Ruler 50 bp DNA ladder, Thermo Fisher Scientific, Lithuania).

### 2.3. Slaughterline examination

The plucks (heart, lung and liver set from each pig) of slaughtered pigs were removed from the slaughterline and visually appraised then palpated for macroscopically visible lesions of pneumonia, pleurisy, pericarditis and liver milk spots according to the Welfare Quality® protocol [11]. A positive case for each pathological lesion was defined as a pig organ affected with any degree of lesion (score 2) and as negative when lesions were absent (score 0). The complete assessment of pathology scores was performed by the three trained investigators.

### 2.4. Welfare indicators

Blood samples were collected in a plastic cup from the bleeding wound at exsanguination and lactate and glucose concentrations were immediately determined in duplicate (two strips/pig) by dipping the test strips into a blood sample. Blood lactate and glucose concentration were measured using a handheld lactate analyzer (Lactate Scout, EKF Diagnostic, Magdeburg, Germany) and glucometer (GlucoSure AutoCode, ApexBio, Taiwan).

*Rigor mortis* intensity was determined on the left carcass side 45 minutes *post-mortem* by measuring the degree of angle between body axis and foreleg according to the Davis *et al.* [12]. For that purpose, photographic images of carcasses were taken, at a distance of approximately 2 m and a height of 160 cm, parallel to the plane in which the carcasses were held. The angle was calculated in AutoCAD program. Angle size and rigor intensity are inversely proportional, e.g. a smaller angle means a higher degree of *rigor mortis*. Subjective assessments of the rigor development were made 45 minutes postmortem on the M. *semimembranosus* in the split carcass using a three-point scale according to [13]: 1) muscle not in rigor; 2) muscle partly in rigor; and 3) muscle in full rigor. Semimembranosus muscle is normally exposed on the carcass medial surface and can be assessed by gauging its surface firmness using finger pressure, whereby in a muscle not in rigor the surface feels soft, while a muscle in rigor feels quite firm.

### 2.5. Growth performance

Average lifetime daily weight gain was derived from live weight at slaughter (carcass weight divided by 0.74 to allow for guts, etc.), minus 1.1 kg (typical weight of newborn piglet), and the total divided by the average age at slaughter, as described by Jaeger *et al.* [14].

### 2.6. Carcass and meat quality analyses

The carcasses were weighed immediately after splitting and final washing to obtain the hot carcass weight, and re-weighed 24 h after chilling at 4°C to determine the cold carcass weight. Backfat and loin muscle (M. *longissimus dorsi*) thickness were measured with a stainless steel ruler on the midline of the split carcass in millimeters: a fat measurement taken as the minimum fat thickness of the visible fat including rind covering the M. *gluteus medius* and a muscle measurement taken at the shortest connection between the front (cranial) end of the M. *gluteus medius* and the upper (dorsal) edge of the vertebral canal. The lean meat content (%) was calculated using ZP (Zwei-Punkte Messverfahren) method [15] based on the thickness of the backfat and loin depth according to the following formula:  $y = 65,93356 - 0,17759 * x_1 + 0,00579 * x_1 - 52,54737 * x_1/x_2$ , with  $y$  = estimated lean meat content of the carcass (kg);  $x_1$  = backfat depth (mm) and  $x_2$  = loin muscle depth (mm). The number of skin lesions on the left side of the carcasses was counted by three trained observers in the chilling room 45 mins *post-mortem* using the Welfare Quality® protocol [11] as described in Čobanović *et al.* [16]. Carcass skin lesions were also classified as human-inflicted type bruises, fighting-type bruises and mounting-type bruises by visual assessment of shape and size to recognize their origin as described in Čobanović *et al.* [16]. The pH and temperature of the M. *longissimus dorsi* (pH<sub>45LD</sub>; T<sub>45LD</sub>) and M. *semimembranosus* (pH<sub>45SM</sub>; T<sub>45SM</sub>) were measured 45 minutes after slaughter using a pH-meter Testo 205 (Testo AG, Lenzkirch, Germany). Pork quality classes (pale, soft and exudative – PSE meat; normal meat; and dark, firm and dry – DFD meat) were determined according to Čobanović *et al.* [16] using pH<sub>45LD</sub> value. The carcasses showing pH<sub>45LD</sub> values lower than 6.0 were classified as PSE meat,

while the carcasses showing pH<sub>45LD</sub> values higher than 6.4 were classified as DFD meat. The carcasses with pH<sub>45LD</sub> between 6.0 and 6.4 were classified as normal pork quality.

### 2.7. Statistical analysis

Statistical analysis of the results was conducted with SPSS software version 23.00 for Windows. According to RYR1 genotype, the pigs were allocated to two groups: Nn = stress-carriers (n=21) and NN = stress-resistant pigs (n=39). Student's t-test was used to examine the effects of RYR1 gene mutation on the growth performance, welfare indicators, and carcass and meat quality traits of slaughter pigs. Data were described by descriptive statistical parameters as the mean value and standard error of the mean (SE). The effects of RYR1 gene mutation on health indicators, type of carcass lesions and pork quality classes were determined by Fisher's exact test. A value of  $P < 0.05$  was considered significant.

## 3. Results and Discussions

Of the 60 examined slaughter pigs, 65.00% contained the normal RYR-1 genotype (NN genotype), 35.00% individuals contained stress-susceptible n allele (Nn genotype), while none of the animals had a double recessive RYR-1 genotype (nn genotype).

Effects of RYR1 genotype on health and welfare indicators in slaughter pigs are shown in Table 1. Stress-carrier pigs were more affected ( $P < 0.05$ ) by pneumonia than stress-resistant pigs. Pig producers and meat companies aim to produce pigs with high meatiness and good meat quality traits at the same time. However, although the genetic selection of pigs significantly improved meatiness, they became less resistant to diseases [17-18], which can explain a higher predisposition for the occurrence of pathological lesions in stress-carrier pigs observed in this study. In stress-carrier pigs, stressful situations presumably induce changes in the number and proportions of white blood cells, mitogen-induced cell proliferation, natural killer cell cytotoxicity and circulating inflammatory factors, plus suppression of cytokines and immunoglobulin production, which can result in increased susceptibility to any infectious disease [19]. Moreover, in the case of an existing disease, stressful factors might predispose Nn pigs to the emergence of various diseases that further complicate the process [19].

**Table 1.** Effects of RYR1 genotype on health and welfare indicators in slaughter pigs

RYR1 genotype	Nn	NN	P-value	Significance
Number of pigs	21	39		
<i>Pathological lesion</i>				
Pneumonia (%)	61.90 <sup>a</sup>	28.21 <sup>b</sup>	0.0145	*
Pleurisy (%)	28.57	23.08	0.7569	NS
Liver milk spots (%)	38.10	35.90	>0.9999	NS
Pericarditis (%)	9.52	7.69	>0.9999	NS
<i>Blood parameters</i>				
Lactate (mmol/l)	12.64 <sup>a</sup> ±1.99	7.86 <sup>b</sup> ±0.74	0.0125	*
Glucose (mmol/l)	9.18 <sup>a</sup> ±0.99	5.13 <sup>b</sup> ±0.39	0.0001	*
<i>Rigor mortis</i>				
Foreleg angle (°)	115.20 <sup>a</sup> ±0.78	120.80 <sup>b</sup> ±0.59	<0.0001	*
Rigor scores	1.05 <sup>a</sup> ±0.15	0.49 <sup>b</sup> ±0.09	0.0010	*

Nn = stress-carrier; NN = stress-resistant; \* Statistical significance at ( $P < 0.05$ ); NS: not significant ( $P > 0.05$ ); <sup>a, b</sup> Different letters in the same row indicate a significant difference at  $P < 0.05$

In this study, higher ( $P < 0.05$ ) blood lactate and glucose concentrations, more developed *rigor mortis*, as well as higher rigor scores were recorded in Nn pigs, which confirmed that the heterozygous individuals containing n allele are more sensitive to stress [20]. Genetic selection of pigs for improved meatiness has led to increased numbers of white muscle fibers that are extremely rich in glycogen,

which could explain 2-3 times higher glycogen contents in stress-carrier pigs than in stress-resistant pigs [21], and, therefore, great potential for accumulating lactate and glucose in the circulation after exposure to stress factors. The mechanism of stress in Nn pigs involves an unusual sympatho-adrenomedullary system response, resulting in increased release of catecholamines (adrenaline and noradrenaline) that bind to specific  $\beta$ -receptors on the cell membrane of skeletal muscles, activating the endocyclase enzymes, and thereby causing depletion of muscle glycogen. This contributes to the increased blood lactate and glucose concentrations and rapid development and higher intensity of *rigor mortis* after exposure to stressful circumstances on the day of slaughter [13, 20].

Effects of RYR1 genotype on growth performance, carcass and meat quality traits in slaughter pigs are shown in Table 2. Nn pigs had higher ( $P<0.05$ ) average lifetime daily weight gain, live, hot and cold carcass weights than NN pigs. In addition, Nn pigs had lower ( $P<0.05$ ) backfat thickness, but higher ( $P<0.05$ ) loin muscle thickness and lean meat content, which confirmed the positive influence of the recessive n allele on the carcass quality [22].

**Table 2.** Effects of RYR1 genotype on growth performance, carcass and meat quality traits in slaughter pigs

RYR1 genotype	Nn	NN	P-value	Significance
Number of pigs	21	39		
<i>Growth performance</i>				
Average lifetime daily weight gain (kg)	0.660 <sup>a</sup> ±0.01	0.596 <sup>b</sup> ±0.01	<0.0001	*
<i>Carcass quality parameters</i>				
Live weight (kg)	114.90 <sup>a</sup> ±1.70	109.90 <sup>b</sup> ±0.73	0.0026	*
Hot carcass weight (kg)	94.86 <sup>a</sup> ±1.66	90.13 <sup>b</sup> ±0.69	0.0032	*
Cold carcass weight (kg)	93.31 <sup>a</sup> ±1.66	88.10 <sup>b</sup> ±0.72	0.0014	*
Backfat thickness (mm)	10.86 <sup>a</sup> ±1.57	14.75 <sup>b</sup> ±0.98	0.0316	*
Loin muscle thickness (mm)	67.33 <sup>a</sup> ±1.30	67.03 <sup>b</sup> ±1.43	0.8876	NS
Lean meat content (%)	56.71 <sup>a</sup> ±1.00	51.99 <sup>b</sup> ±0.80	0.0007	*
Skin lesion score	0.52 <sup>a</sup> ±0.15	1.03 <sup>b</sup> ±0.13	0.0224	*
<i>Type of skin lesions (%)</i>				
Human-inflicted bruises	38.10 <sup>a</sup>	10.26 <sup>b</sup>	0.0169	*
Fighting-type bruises	4.76 <sup>a</sup>	43.59 <sup>b</sup>	0.0024	*
Mounting-type bruises	0.00	12.82	0.1519	NS
<i>Meat quality parameters</i>				
pH <sub>45LD</sub>	6.01 <sup>a</sup> ±0.03	6.36 <sup>b</sup> ±0.03	<0.0001	*
T <sub>45LD</sub>	37.23 <sup>a</sup> ±0.07	36.52 <sup>b</sup> ±0.05	<0.0001	*
pH <sub>45SM</sub>	6.07 <sup>a</sup> ±0.07	6.30 <sup>b</sup> ±0.02	0.0002	*
T <sub>45SM</sub>	37.07 <sup>a</sup> ±0.06	36.45 <sup>b</sup> ±0.04	<0.0001	*
<i>Pork quality classes (%)</i>				
PSE meat	42.86 <sup>a</sup>	2.56 <sup>b</sup>	0.0002	*
Normal meat	57.14 <sup>a</sup>	84.62 <sup>b</sup>	0.0289	*
DFD meat	0.00	12.82	0.1519	NS

Nn = stress-carrier; NN = stress-resistant; \* Statistical significance at ( $P<0.05$ ); NS: not significant ( $P>0.05$ ); <sup>a, b</sup> Different letters in the same row indicate a significant difference at  $P<0.05$

The results obtained in this study could be explained by the facts that Nn pigs have a more favorable daily feed intake, increased feed conversion efficiency, and, consequently, higher growth rate and meatiness compared to NN pigs [7,22]. Better feed utilization and the higher carcass lean content could be attributed to the faster metabolism, as well as greater ability of stress-carrier pigs to assimilate proteins, and a lower predisposition for fat deposition [22]. In the present study, NN pigs

had greater ( $P<0.05$ ) overall skin lesion score and fighting-type bruises than Nn pigs (Table 2). The higher predisposition of stress-resistant pigs to severe skin lesions could be explained by intense exploratory behavior, which increases contact with pen mates and resulting in confrontations and fights between pigs [23]. In contrast, Nn pigs exhibit greater fear and less curiosity to explore, and, therefore, rarely come into conflict with pen mates [23]. However, the frequency of carcass lesions caused by rough handling was higher ( $P<0.05$ ) in Nn pigs (Table 2), which could be preferentially attributed to their greater sensitivity to stressful stimuli [23]. Due to the higher sensitivity to stress and, consequently, the greater anxiety of Nn pigs, they were more difficult to handle and needed more force, as well as excessive use of sticks and electric prods at loading, unloading and through the lairage raceways, which resulted in a higher frequency of carcass lesions indicating rough handling during the pre-slaughter period [23].

In this study, stress-carrier pigs had lower pH and higher temperature values measured 45 mins *post-mortem* in both muscles, as well as a higher prevalence of PSE meat ( $P<0.05$ ; Table 2). Conversely, stress-resistant pigs had a higher ( $P<0.05$ ; Table 2) prevalence of normal meat. Although skeletal muscles from pigs containing the mutant n allele (Nn genotype) have lower than normal contractile thresholds and an enhanced sensitivity as a consequence of abnormal intracellular calcium homeostasis [6], under normal conditions, the muscles function undisturbed [1]. However, exposure of stress-carrier pigs to stressful conditions just prior to slaughter leads to increased accumulation of calcium ions due to a genetic mutation in the skeletal muscle calcium release channel. Once opened, the mutated channel is unresponsive to  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -induced closing, thereby provoking muscle contracture, hypermetabolism and hyperthermia [1]. This causes extreme muscle glycogen depletion, resulting in a rapid fall in meat pH, which in combination with high carcass temperature leads to the myofibrillar and sarcoplasmic protein denaturation, subsequently affecting water-holding capacity of pork, which increasing the tendency towards PSE meat [3-5,7].

#### 4. Conclusion

The results of this study showed that pigs containing the mutant n allele (Nn genotype) produced better carcass quality (higher live weight, carcass weight and meatiness, but less fat). However, the presence of the mutant n allele in these pigs had a deleterious effect on animal welfare and meat quality showed by the increased blood lactate and glucose concentrations, more developed *rigor mortis* and higher prevalence of PSE meat. In addition, pigs containing the mutant n allele (Nn genotype) had a higher predisposition for pneumonia, indicating that they are more prone to infectious diseases. Accordingly, further selection towards the elimination of the mutant allele of the RYR1 gene from pig populations would result in an improvement of health, welfare, and pork quality.

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## Protective effect of *Gentiana lutea* root and leaf extracts against heterocyclic aromatic amines IQ and PhIP produced in thermally processed meat

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**Abstract.** During high-temperature cooking of protein rich foods, especially meat and fish, heterocyclic aromatic amines can be formed. These amines are a class of potent mutagens that can cause alterations in the structure of DNA and chromosomes. In recent decades, research has been focused on investigating plants and their phytochemicals as potential antimutagens. The aim of this study was to examine the anti-genotoxic effect of methanolic root and leaf extracts of *Gentiana lutea* against the food mutagens 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) produced in thermally processed meat. To determine the protective potential of extracts, the alkaline comet assay was applied. The results obtained indicated strong anti-genotoxic effect of both extracts against the tested mutagens. The highest inhibition of IQ-induced genotoxicity was recorded for leaf extract (72%). Regarding PhIP, root extract achieved inhibition of 80% of DNA damage, so was more successful than leaf extract. The data obtained in this study stimulates further research of *G. lutea* extracts and its constituents as potential dietary supplements in improving human health.

### 1. Introduction

Diet can contribute to an increased risk of cancer development, due to the consumption of food mutagens. Food mutagens cause different types of damage in DNA molecule, specifically nucleotide alterations and chromosomal aberrations, by forming carcinogen-DNA adducts. An important class of compounds that are considered a dietary risk factor for development of cancer are heterocyclic aromatic amines (HAAs) [1]. HAAs are formed during the high-temperature cooking of protein rich foods, especially meat and fish, and can significantly increase the risk of different cancers, mainly colon cancer [2]. Numerous studies have been carried out in recent decades in order to identify compounds that might be able to reduce DNA damage. Plants are a rich source of various phytochemicals that can be beneficial for human health, including protection against HAA-induced genotoxicity.

Plants from the genus *Gentiana* are well known for their various biological activities, including antioxidant, antimicrobial, anticancer and radioprotective properties. *Gentiana lutea*, yellow gentian, is widely used in traditional medicine, as well as in the food and pharmaceutical industries. Yellow



gentian root is an officinal drug in many pharmacopoeias for the treatment of mild gastrointestinal diseases [3,4,5].

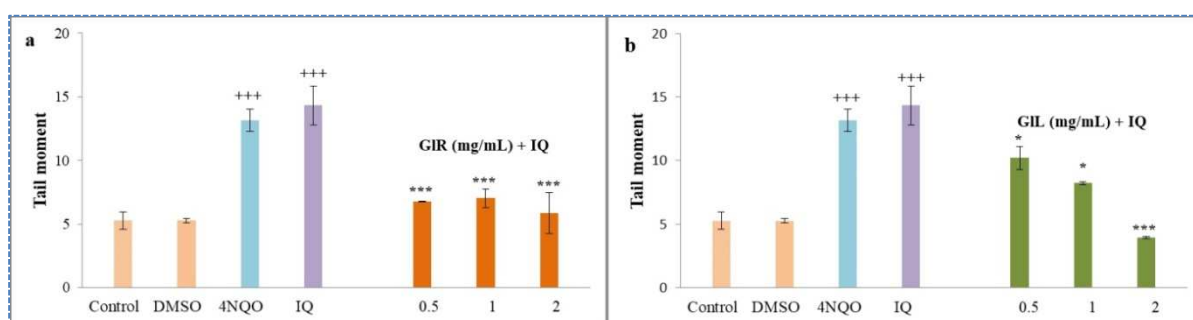
Therefore, the aim of this study was to examine the anti-genotoxic potential of *G. lutea* methanolic root and leaf extracts against 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) mutagens produced in thermally processed meat.

## 2. Materials and Methods

Plant material was obtained from the Institute for Medicinal Plants Research Dr Josif Pančić, Belgrade, Serbia. Extract were prepared as previously described by Nastasijević et al. [6]. Chemical characterization of extracts was performed by Ultra Performance Liquid Chromatography (UPLC) as described previously [6]. Results were calculated according to dry weights of root/leaf extract. In order to determine non-cytotoxic concentrations of extracts and food mutagens, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed as previously described by Vasiljević et al. [7]. Genotoxicity and anti-genotoxic potential of extracts and mutagens were tested using the alkaline comet assay as performed by Mitić-Ćulafić et al. [8]. Human hepatocellular carcinoma cells (HepG2) were used as a model system for testing the biological activities. For anti-genotoxicity testing, cells were exposed to co-treatment of extracts and mutagens for 24 h. The statistical analysis was carried out using Mann-Whitney *U* test. Kolmogorov-Smirnov test was used to determine if data were normally distributed. Inhibition of IQ- and PhIP-induced genotoxicity was calculated using the following formula:  $I (\%) = 1 - (Nt/Nc) \cdot 100$ , where *Nt* is the mean value of tail moment of co-treated groups; *Nc* is the mean value of tail moment of IQ/PhIP.

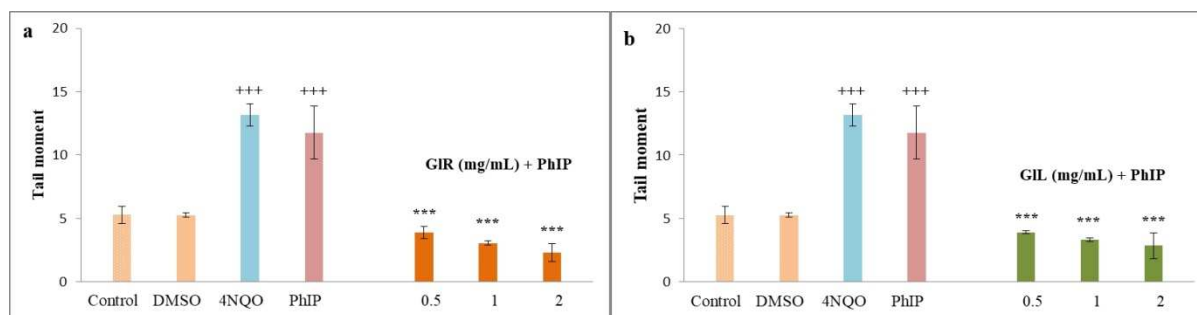
## 3. Results and Discussion

Results of UPLC showed the most abundant constituents present in the extracts were gentiopicroside ( $2.4 \pm 0.2\%$ ) and loganic acid ( $0.18 \pm 0.01\%$ ) in root and leaf extracts, respectively. Preliminary cytotoxicity testing determined the non-cytotoxic doses of extracts and food mutagens: up to 2 mg/mL for extracts; up to 200  $\mu\text{g/mL}$  for mutagens. The highest non-cytotoxic concentrations of extracts and mutagens were tested for genotoxicity to establish the doses of extracts that are non-genotoxic on one hand, and to determine the genotoxic dose of mutagens that induced sufficient DNA-damage, on the other hand. The protective effect of the extracts was tested against IQ (200  $\mu\text{g/mL}$ ) and PhIP (100  $\mu\text{g/mL}$ ). Results of anti-genotoxicity testing are shown in Figures 1 and 2.



Results are presented as mean values of tail moment  $\pm$  SD; GIL – *G. lutea* root extract; GIL – *G. lutea* leaf extract; 4-Nitroquinoline N-oxide (4NQO)-positive control (10 $\mu\text{M}$ ); \*\*\*Significant differences between co-treated groups and mutagen; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; +++Significant differences in regard to dimethyl sulfoxide (control solution); + $p < 0.05$ ; ++ $p < 0.01$ ; +++ $p < 0.001$

**Figure 1.** Anti-genotoxic potential of *G. lutea* root (a) and leaf (b) extracts against IQ-induced genotoxicity



Results are presented as mean values of tail moment  $\pm$  SD; GIR – *G. lutea* root extract; GIL – *G. lutea* leaf extract; 4-Nitroquinoline N-oxide (4NQO)-positive control (10 $\mu$ M); \*\*\*Significant differences between co-treated groups and mutagen; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; +++Significant differences in regard to dimethyl sulfoxide (control solution); + $p$ <0.05; ++ $p$ <0.01; +++ $p$ <0.001

**Figure 2.** Anti-genotoxic potential of *G. lutea* root (a) and leaf (b) extracts against PhIP-induced genotoxicity

Exposure of HepG2 cells to IQ and PhIP for 24 h induced significant increase in DNA damage as shown in Figures 1 and 2. The IQ-induced genotoxicity was significantly reduced in the presence of *G. lutea* root extract. Similarly, leaf extract exhibited strong anti-genotoxic potential especially at the highest concentration (2mg/mL) with the inhibition of IQ-induced DNA damage being 72% (Figure 1b, Table 1). Furthermore, both root and leaf extract prevented PhIP-induced DNA strand breaks significantly at all tested concentrations. The highest inhibition of genotoxicity (80%) was recorded for the root extract at the concentration of 2 mg/mL (Table 1)

**Table 1.** Inhibition of IQ- and PhIP-induced genotoxicity

	IQ			PhIP		
mg/mL	0.5	1	2	0.5	1	2
GIR	53%	51%	58%	67%	73%	80%
GIL	29%	43%	72%	66%	72%	76%

GIR – *G. lutea* root extract; GIL – *G. lutea* leaf extract

Taking into account that HAAs can express mutagenic potential at ng/g levels in cooked foods and can consequently play an important role in the aetiology of human cancer, it is very important to find a way to reduce their harmful effect. Using plants and their phytochemicals as potential protective agents in improvement of human health is a current trend in recent years.

The results obtained in this study are in accordance with available literature data. Viegas et al. [9] showed that flavonoid xanthohumol, present in *Humulus lupulus*, exhibited the complete prevention of PhIP-induced DNA damage. Furthermore, significant protective effect of flavonoids quercetin and rutin was demonstrated after DNA damage induced by IQ and PhIP [10]. Rosemary extracts were also effective in the prevention of DNA strand breaks induced by PhIP [11].

A possible explanation for the protective role of *G. lutea* extracts against HAAs may lie in the fact that yellow gentian is known for its antioxidant properties. In previous studies, root extracts expressed quite strong antioxidant activity, with the IC<sub>50</sub> value at 20.6  $\mu$ g/mL recorded in the 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assay [6, 12]. Considering the fact that aside from forming the DNA-adducts, HAAs might also generate reactive oxygen species that cause oxidative DNA damage [13,14], using compounds with strong antioxidant potential is a promising way to protect the integrity of genome.

#### 4. Conclusions

The results obtained in this study point to methanolic root and leaf extracts of *G. lutea* as potential protective agents against the thermally produced food mutagens, IQ and PhIP. Both extracts expressed statistically significant anti-genotoxic effects at all tested concentrations. The highest inhibition of IQ- and PhIP-induced genotoxicity was recorded for leaf extract (72%) and root extract (80%), respectively. The data obtained are promising, indicating yellow gentian is suitable as a potential dietary supplement to improve human health. In accordance with our results, further research should be focused on investigating the protective effect of *G. lutea* extracts and constituents in *in vitro* and *in vivo* model systems.

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# Quality of meat products from the Serbian market in terms of protein content

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**Abstract.** The quality of meat products on the market constantly comes into question. In Serbia, there remains some level of persistent distrust and suspicion regarding meat processors and meat quality. Protein quality, meaning protein content and percentage of collagen in total protein, is chemical quality parameter stipulated by regulation. Forty meat products from the Serbian retail market, of which 19 were declared, by Serbian regulation, as canned meat in pieces and 21 were declared as finely ground cooked sausages (emulsion-type, pasteurised hotdogs), were investigated. All hotdogs (100%) fulfilled the required protein quality criteria, while 36% of canned meat in pieces were not in accordance with the regulation regarding protein quality. The main reason for products not meeting the regulation was their high collagen levels. In total, 15% of the meat products studied did not meet legal protein requirements.

## 1. Introduction

Meat and meat products are important protein sources in the human diet. Meat consumption depends on economic factors, religion, ethics and tradition. In Serbian cuisine, meat and meat products have a special place, a long tradition of consumption, and are normally an essential part of at least one meal per day [1, 2]. In a household survey from 2017, it was estimated that each household in Serbia spent the most of its food budget on meat and meat products, exactly 5952 RSD per month. Also, it was noted that average household consumption of meat products for 2017 was 38.9 kg, i.e. 3.24 kg per month [3]. Because of the high meat consumption level in Serbia, consumers demand high quality meat and meat products.

Hotdogs are declared by the “Regulation of the quality of ground meat, meat preparations, semi prepared meat products and meat products” to be finely ground cooked sausages, and are one of the most commonly consumed meat products, because of their low salt content and soft texture. Canned meat in pieces are another subgroup defined by the regulation. Most pizza hams and chicken breast in casings are declared as canned meat in pieces. They are very popular among consumers with special diet regimes because of their low-fat content [4, 5].

In Serbia, as in other countries, the quality of meat and meat products on the market is a constantly debated theme. Hence, the objective of this study was to investigate the protein content and percentage



of collagen, as chemical quality parameters defined by regulation, in hotdogs and canned meat in pieces, to see if meat processors meet the regulation and if consumers are protected from food fraud.

## 2. Materials and methods

### 2.1. Meat products sampled

Commercially available hotdogs (HD) and canned meat in pieces (CM), both made of chicken meat or pork, were collected from the Serbian retail market during 2018. Hotdogs (n=21), of which 11 were made of chicken meat and 10 of pork, and canned meat in pieces (n=19), of which 11 were made of chicken meat and 8 of pork, were collected. The meat products were produced by the most common meat processors in the Serbian retail market. After collection, meats were homogenized and stored at -18°C until analysis. All analyses were conducted in triplicate.

### 2.2. Total protein content analysis

Total protein content was determined according to the Kjeldahl method recommended by International Organization for Standardization [6].

### 2.3. Collagen share

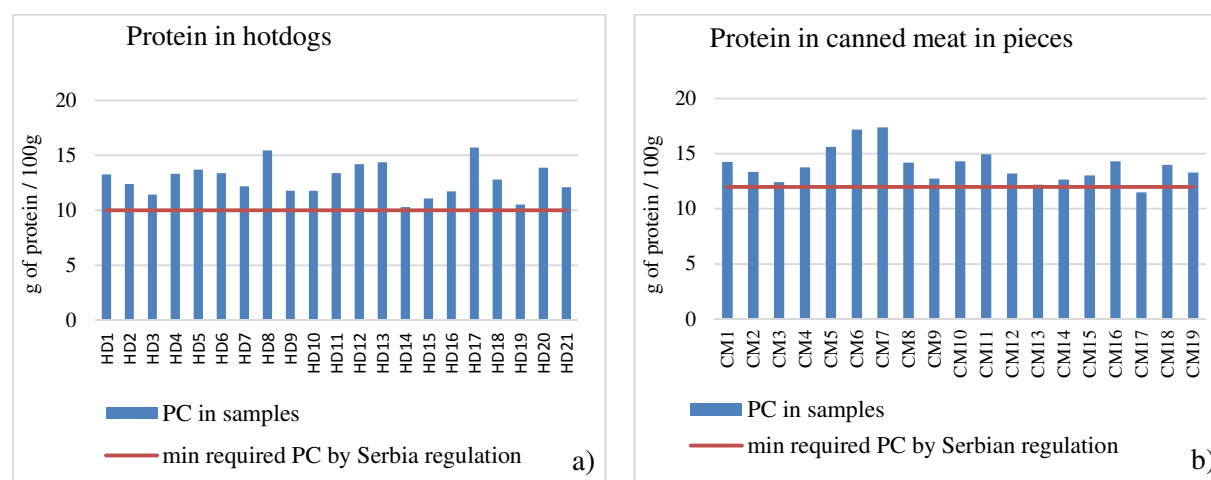
Collagen content was calculated by multiplication of hydroxyproline content by a factor of 8, while hydroxyproline content was determined by method SRPS 2002, ISO 3496 [7]. Collagen was expressed as the percent of collagen in the total protein content.

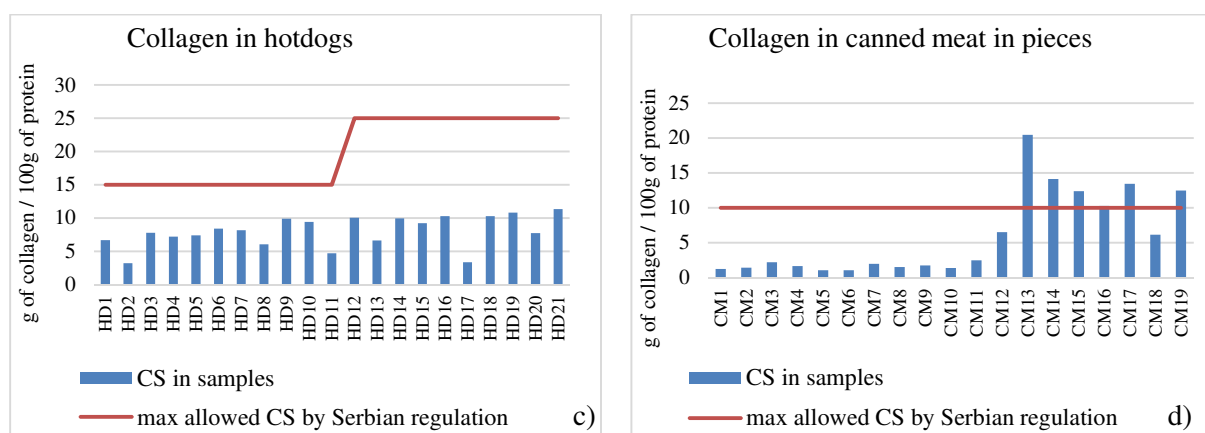
### 2.4. Statistical analysis

The experimental data were analysed using the two-way T-test for single samples (test of means against a reference constant) with significance level of 95% ( $p < 0.05$ ). The tests were performed by Statistica version 13 [8].

## 3. Results and discussion

The mean total protein content and percent of collagen (in the total protein content) in the analysed HD and CM, as well as protein content and percent of collagen required by Serbian law are presented in figure 1. The current regulation requires minimum 10% protein and maximum 25% collagen in pork HD and 15% collagen in chicken HD. For CM, the legal limits are minimum of 12% protein and maximum 10% collagen [5].





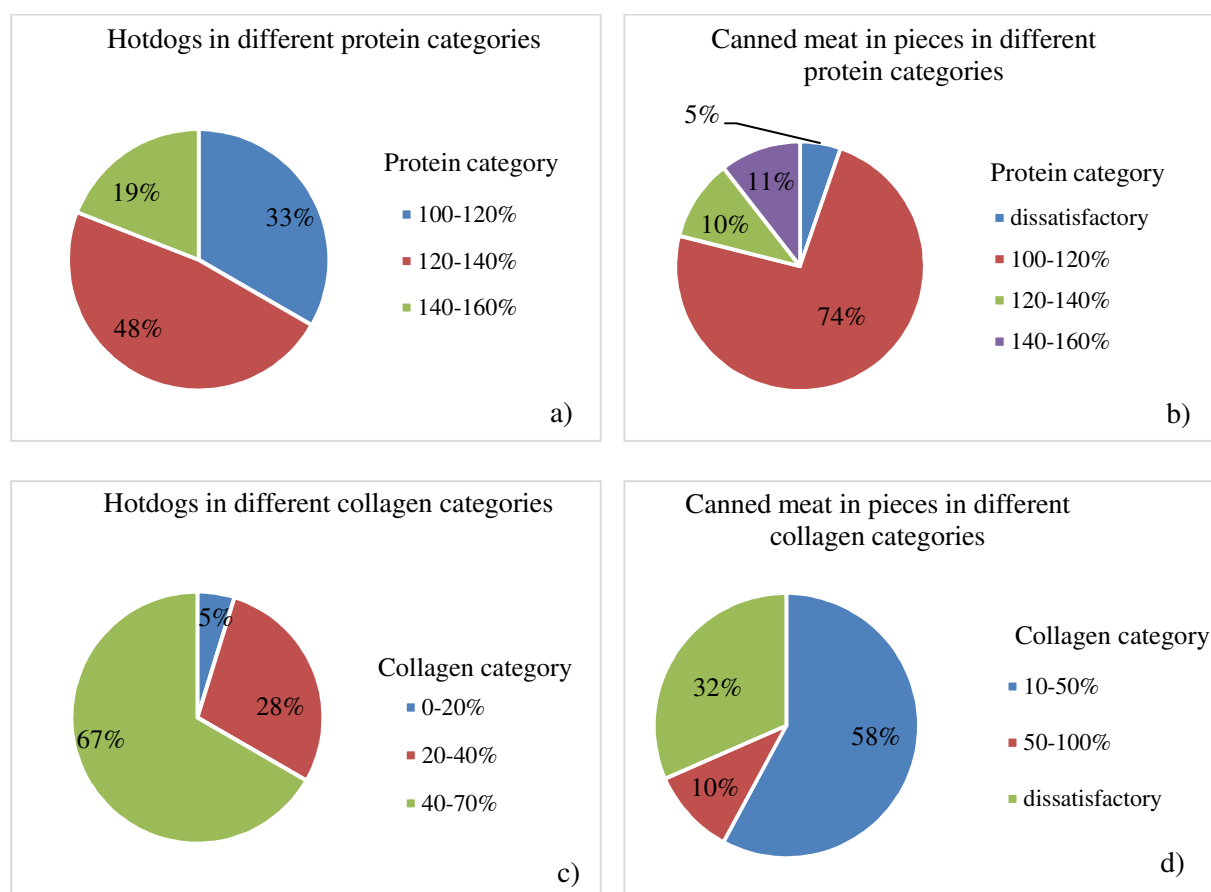
**Figure 1.** The mean total protein content (PC) and collagen share (CS) of analysed meat products and values required by regulation: a) the PC in hotdogs; b) the PC in canned meat in pieces; c) the CS in hotdogs; d) the CS in canned meat in pieces

The protein content in HDs ranged from 10.31 (HD14) to 15.70 (HD17) g/100g. The collagen in HDs ranged from 3.22 (HD2) to 11.33 (HD21) g/100g of protein. In CMs, the lowest protein content detected was 11.48 (CM17) g/100g, while CM7 contained the highest protein level, 17.40 g/100g. The collagen in CMs ranged from 1.05 (CM5) to 20.45 (CM13) g/100g of protein. However, figure 1d shows some CMs did not fulfil regulatory requirements for collagen content. Furthermore, the range of collagen contents in the CMs was very wide. The first part of the CM group were labelled as chicken breast (*pileća prsa*), and second part, which had high collagen contents, were labelled as pizza ham (*pizza šunka*). Hence, the chicken breast CMs met the regulatory requirements for collagen. It is possible that since pizza ham does not usually command a high price, more connective tissue than is recommended was used in these products.

Figure 2 shows the percentages of HDs and CMs that contained different levels of protein and collagen. Protein and collagen levels were calculated as the percentage of the levels required by regulation (100%). All means of measured values (protein content and % collagen) were significantly different (lower or higher) to their regulated criteria ( $p < 0.05$ ). All HDs ( $n=21$ ; 100%) met the regulatory requirements, and had higher protein content than the minimum required by law, and lower % collagen than the maximum % collagen stipulated by law. Furthermore, a large group of HDs, 48% in fact, contained 120-140% of the protein stipulated by law. Collagen contents in all HDs were in accordance with regulation. Moreover, all HDs contained less than 70% of the maximum allowed % collagen. In 2006, Saicic et al. examined 85 finely ground cooked sausages. Among these 85 cooked sausages, 14 (16.47%) did not satisfy legal criteria [9]. Furthermore, Kurcubic et al. reported that of 123 finely ground cooked sausages, 47 (27.65%) did not meet the regulation, of which 21 (17.07%) sausages had lower protein content and 33 (26.83%) had higher % collagen [10]. From the results presented in this paper, it seems likely that current production of finely grounded boiled sausages (hotdogs) has prospered and that meat processing industries have stopped using excessive amounts of connective tissue.

However, analysis of CMs showed these products did not always comply with the regulation. The protein content in one CM (5%) was too low and did not accord with the regulation. Furthermore, 6 CMs (32%) contained unsuitably high % collagen, i.e. over the maximum allowed level. However, 74% of CMs had protein contents in the range of 100-120% of the legal minimum, and 58% of CMs had % collagen less than 50% of the maximum allowed level. The data show collagen content was the most common reason the meat products were not in accordance with regulation.





**Figure 2.** The percentages of meat products with different protein and collagen contents as a percentage of the levels required by regulation: a) Percentages of hotdogs in protein categories; b) Percentages of canned meat in pieces in protein categories; c) Percentages of hotdogs in collagen categories; d) Percentages of canned meat in pieces in collagen categories

#### 4. Conclusion

In conclusion, meat processors followed the regulatory requirements for protein and collagen when producing HDs. All the HDs (100%) had protein and collagen contents in accordance with stipulated Serbian law. In the case of CMs, six samples (32%) were not in accordance with regulation. However, among all 40 meat products examined (HD and CM) only six (15%) did not satisfy the regulatory criteria, mostly because of abundant usage of connective tissue. From previously published results of protein quality in meat products on the Serbian market and the results presented in this work, it seems the quality of hotdogs and canned meat in pieces on the Serbian market is improving.

#### Acknowledgement

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## Phosphates as food additives in meat and meat products in North Macedonia

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**Abstract.** Phosphates are used as food additives by meat processing industries to improve the technological properties of the products. However, phosphates can have adverse effects on human health so their use is regulated by law setting the maximum allowable level of phosphates in a meat product. This study evaluates the total phosphorus contents in meat and meat products placed on the Macedonian market in the previous five years. The results showed that a significant number of analysed meat products contained more than 5000 mg/kg total phosphorus as P<sub>2</sub>O<sub>5</sub>. However, it must be stressed that for the time being there is no analytical methodology available capable of distinguishing the naturally present phosphates from the added ones.

### 1. Introduction

Phosphorus is essential for all living organisms. Inorganic phosphate used as food additive is assumed to dissociate in the gastrointestinal tract. Approximately 80-90% of the inorganic phosphorus deriving from food additives is absorbed as free orthophosphate. Excretion is *via* the kidney through glomerular filtration and tubular mechanisms. The EFSA panel on food additives and flavourings considered phosphates to be of low acute oral toxicity, and to be of no concern with respect to genotoxicity and carcinogenicity [1]. In standard short-term, subchronic and chronic toxicity studies, the only significant adverse effects of phosphates are calcification of the kidney and tubular nephropathy. The epidemiological studies recently reviewed did not find consistent associations between dietary phosphorous intake and cardiovascular-related outcomes and do not provide sufficient and reliable data to assess the role of phosphate in bone health. Clinical interventional trials in which phosphorous doses were administered on top of normal diets were performed over several months. No impairment of renal function was reported with daily doses of up to 2000 mg phosphorus (28.6 mg/kg per day), whereas doses of 4800 mg (68.6 mg/kg per day) elicited renal impairment [1].

The EFSA panel concluded a group acceptable daily intake (ADI) of 40 mg/kg body weight per day, expressed as phosphorus, is protective for healthy adults because it is below the doses at which clinically relevant adverse effects were reported in short-term and long-term studies in humans. However, this ADI does not apply to people with moderate to severe reduction in renal function [1].



Phosphates used in meat processing industries are salts of phosphoric acid, sodium or potassium. Phosphates are polyvalent ions which can form structures containing from one to hundreds or even thousands of phosphate tetrahedra. Depending on the number of P atoms in the molecule, the usual name will change as follows: (i) one phosphorus atom ( $\text{PO}_4$ )<sup>3-</sup> monophosphates (formerly orthophosphates); (ii) two phosphorus atoms ( $\text{P}_2\text{O}_7$ )<sup>4-</sup> diphosphates (formerly pyrophosphates); (iii) three phosphorus atoms ( $\text{P}_3\text{O}_{10}$ )<sup>5-</sup> tripolyphosphates; and more than three phosphorus atoms ( $\text{P}_n\text{O}_{3n+1}$ )<sup>(n+2)-</sup> polyphosphates [3,4].

Phosphates are authorised food additives in the EU in accordance with Annex II and III to Regulation (EC) No 1333/2008. According to the European legislation, food phosphates are not permitted in fresh meat, but can be added at a maximum amount of 0.5 % (expressed as  $\text{P}_2\text{O}_5$ ) to meat products [5].

Food phosphates used in meat and meat products must be manufactured according to good manufacturing practices (GMP). Phosphates are not permitted in fresh meat but can be added to meat preparations, minced meat and meat products (Regulation EC No 853/2004, 2004). The maximum permitted level of phosphates in meat and meat products according to European legislation is 5 g/kg expressed as phosphorus pentoxide ( $\text{P}_2\text{O}_5$ ) individually or in combination in the finished product (Directive No 95/2/EC, Rev. 2006). Accordingly, the national legislation in North Macedonia for food additives, which is harmonised with EU legislation, stipulates the same maximum permitted level for phosphates in meat products, 5 g/kg [6].

Meat processing operators in North Macedonia are obligated according to the food safety law to confirm the safety of their products by appropriate testing conducted by an external laboratory. The Institute of Public Health has developed laboratory capacities for testing phosphates in meat products at the request of food business operators.

The aim of the study was to evaluate the findings from phosphate determinations in raw meat and meat products for the period from 2014 to 2019.

## 2. Materials and methods

Over a period of 5 years (2014-2019), 323 samples of raw meat and meat products were analysed for total phosphorus content. The samples were delivered to the laboratory by the Food and Veterinary Agency of the Republic of North Macedonia under regular surveillance programs or by the manufacturers interested in the quality of raw materials or of their own manufactured meat products. Of the total number of samples, 190 were pork/beef meat products, 109 were poultry meat products, 12 samples of raw beef/pork meat and 12 samples of raw poultry meat.

After receiving the samples in the laboratory, they were homogenised with appropriate equipment, kept in a seal-tight container at 4°C and analysed within 24 h of homogenisation.

The total phosphorus content in the samples was determined by the standard ISO method[7].

## 3. Results and Discussion

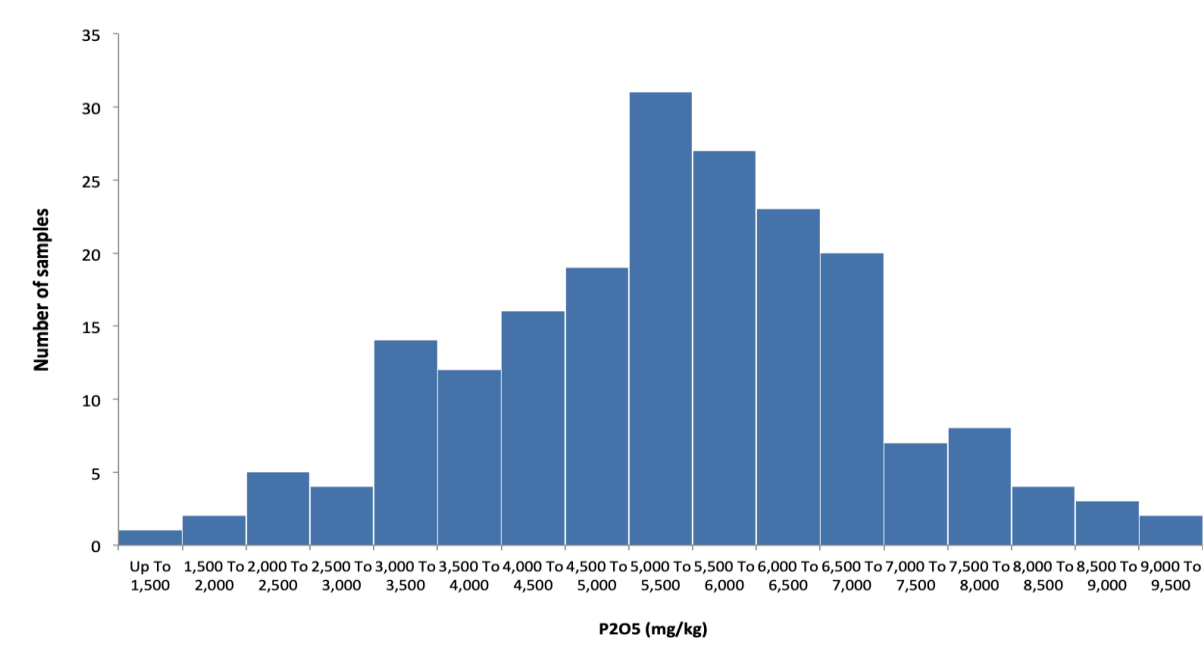
The total phosphorus contents in meat and meat products placed on the North Macedonian market are presented in Table 1.

**Table 1.** Total phosphorus (mean  $\pm$  standard deviation), minimum and maximum contents measured in meat and meat products in North Macedonia, 2014-2019

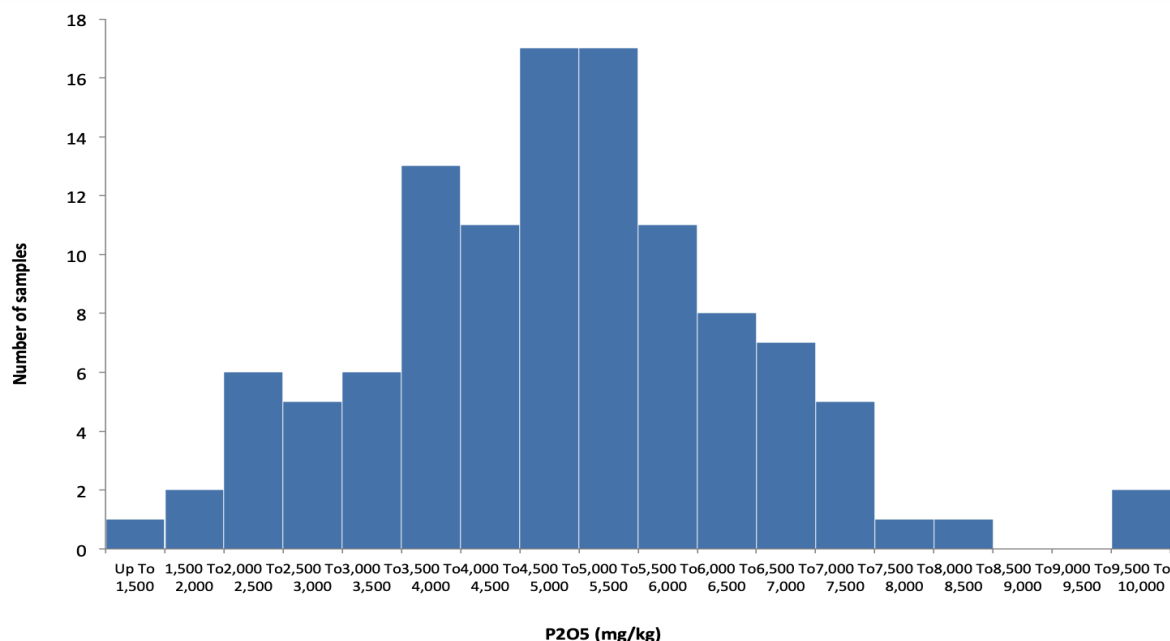
Type of sample	No. of samples	Average $\text{P}_2\text{O}_5$ (mg/kg) content	Minimum content $\text{P}_2\text{O}_5$ (mg/kg)	Maximum content $\text{P}_2\text{O}_5$ (mg/kg)	No. of samples >5000 mg/kg $\text{P}_2\text{O}_5$
Beef/pork meat products	190	5396 $\pm$ 1492	1783	9408	122
Beef/pork raw meat	12	4234 $\pm$ 1312	2298	6038	4

Poultry meat products	109	$4872 \pm 1462$	1878	9737	50
Poultry raw meat	12	$4898 \pm 769$	3474	6268	5

Over 60% of the beef/pork meat products (Figure 1) and around 45% of the poultry meat products (Figure 2) exceeded the legal limit of 5000 mg/kg added phosphates expressed as  $P_2O_5$ , which is a much greater percentage of unsatisfactory products than was published by Prica *et al.* [8]. The mean content of total phosphorus in beef/pork meat products was 5396 mg/kg. The maximum contents of  $P_2O_5$  in meat products were measured in smoked turkey drumstick (9737 mg/kg) and smoked pork tenderloin (9408 mg/kg). Considering raw meat, one sample of frozen pork loin contained 6038 mg/kg  $P_2O_5$ , and the maximum content of  $P_2O_5$  among the raw poultry meats was measured in a sample of chicken steak (6268 mg/kg). The results obtained for poultry meat were similar to those reported by Serdar *et al.* [9].



**Figure 1.** Distribution of the beef/pork meat products according to their  $P_2O_5$  content in mg/kg



**Figure 2.** Distribution of the poultry meat products according to their  $P_2O_5$  content in mg/kg

Elevated values of total phosphorus in raw meat indicate the raw materials used by the meat industry were treated with additives, which is against the law. Moreover, addition of phosphates during the technological process of manufacturing meat products additionally enhances their phosphate contents over the content of phosphate in the initial raw meat used.

Another point of discussion is the limitation of the method used for measurement of the total phosphorus content. This method is incapable of differentiating the naturally present phosphates in the meat and the ones added as food additives in the meat products. As a result, increased values of phosphates were especially encountered in smoked products with high protein content, even though they were produced without addition of phosphates as food additives. An alternative solution of this analytical problem could be determination of the added phosphates by calculation. The protein content of the meat product is used in the formula, based on the approximation that the ratio between the phosphorus content and the meat protein content is constant, as was proposed by Deric *et al.* [10].

The EFSA panel on food additives and flavourings, in their re-evaluation of phosphates, concluded the development of analytical methods for the determination of phosphate additives in the range of foods and beverages permitted to contain them should be considered.

#### 4. Conclusion

A significant number of meat products analysed in this study contained total phosphorus in levels higher than 5000 mg/kg  $P_2O_5$ . The maximum limit for phosphorus set by legislation refers to the added phosphates during technological processing of the meat. On the other hand, no analytical method is able to distinguish between the naturally present phosphates in a meat product and those added as food additives. As a result, the interpretation of the analytical results still remains under debate and requires knowledge of the natural levels of phosphorus in the raw materials used to manufacture the meat products. In any case, it is important to monitor phosphates as additives in meat products and ensure levels are maintained below the maximum limit, in terms of consumer health, but also in terms of quality and safety of the products.

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## Sustainability of animal origin food waste in Serbia

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**Abstract.** This research analysed attitudes related to food waste sustainability and estimated amounts of animal origin food waste and food packaging waste discarded in Serbia. The field survey covered 494 respondents from Belgrade, Serbia. Results present their degree of agreement with nine selected food waste sustainability statements and their reported quantities of discarded animal origin food waste and food packaging waste. Our study showed respondents have a higher rate of agreement related to the negative social and environmental dimensions of food waste, while they have no opinions associated with the economic dimensions. Regarding animal origin food waste quantities, it is estimated that households dispose around 200 g of waste every week (11.3 kg per year) and slightly under 30 different pieces of food packaging. On an annual basis, 7,234 tons of CO<sub>2</sub> emissions can be associated with animal origin food waste and 706.4 tons with food packaging waste in Serbia.

### 1. Introduction

It is a common belief that size of a household influences the amount of food waste discarded [1]. One of the main reasons is that different household members prefer different types of food and consequently produce more food waste [2]. However, when it comes to household members, homes with more members actually produce less waste per capita [3]. Therefore, four household factors that determine food waste are size, income, demographics and culture [4]. When it comes to determination of factors that affect food waste performance from purchasing to eating at home, Schanes et al. [5] point to the following: purchase planning and patronage, food storage, cooking habits, eating and managing leftovers. In consumer societies, various exaggerations influence food waste such as overbuying [6], purchasing oversized packaging [2] and cooking too much [7].

Sustainability as a modern concept consists of three pillars – social, environmental and economic [8]. The food industry recognizes “zero waste” as a food waste management idea that is overseen as an environmental objective leading to a more sustainable society and economy [9]. Therefore, besides the environmental dimension, food waste also has social and economic impacts [10]. Finally, looking at the Sustainable Development Goals [11], food waste is recognized as a hot topic where target 12.3 aims to halve per capita global food waste at retail and consumer levels and reduce food losses by 2030 [12].

The aim of this study was to analyse respondents’ sustainability beliefs related to food waste in Serbia and to calculate the amount of household wastes deployed from different animal origin foods and types of related food packaging. Also, we quantified CO<sub>2</sub> emissions from animal origin food waste and food packaging waste.



## 2. Materials and methods

Field research on food waste from the households in Serbia was performed during the first half of 2018 covering 494 questionnaires that were further processed [13]. The questionnaire was created taking into account published research on food waste [14-17].

Attitudes related to food waste were rated using a five-point Likert scale from 1 “strongly disagree”, 2 “disagree”, 3 “no opinion”, 4 “agree” to 5 “strongly agree”. Also, respondents were requested to indicate the frequency and the amount of food groups discarded in the last seven days, and the animal origin of the food, namely (i) fish and/or meat, or (ii) dairy products, which were further analysed. Animal origin food packaging waste generated in households was deployed in terms of tetra-pak packaging (associated with dairy products such as milk and yoghurt) or polyethylene films and paper (wrappers) used for packaging cheese, meat and/or meat products. Four frequency options were available as follows: every day (recorded as 7 times), 3 times per week (recorded as 3), 2 times per week (recorded as 2), and once a week (recorded as 1). Quantity of food waste was evaluated in handfuls of food waste, estimated as 20g or 20mL [13] or “zero” if they did not consume/discard this type of waste. Quantity of food packaging waste was evaluated in units of packaging or “zero” if they did not purchase/discard food with this type of waste. Weight measurement of typical representatives of each type of food packaging was performed at the Faculty of Agriculture with the following data (tetra-pak packaging – 27.0 g, paper and plastic wrappers – 2.5 g).

In order to calculate the global warming potential (GWP) associated with this type of food waste and packaging, the following assumptions were applied: (i) bearing in mind that food wastes from this urban city (Belgrade) are disposed at municipal landfills with no recycling, emission production was estimated as 0.513 kg CO<sub>2</sub>/kg food waste; (ii) tetra-pak packaging – 0.045 kg CO<sub>2</sub>/kg food packaging waste; (iii) plastic wrappers – 0.071 kg CO<sub>2</sub>/kg food packaging waste; (iv) paper wrappers – 0.035 kg CO<sub>2</sub>/kg food packaging waste. GWP data were extracted from ©CCaLC and Ecoinvest databases [18].

## 3. Results and discussion

### 3.1. Demography

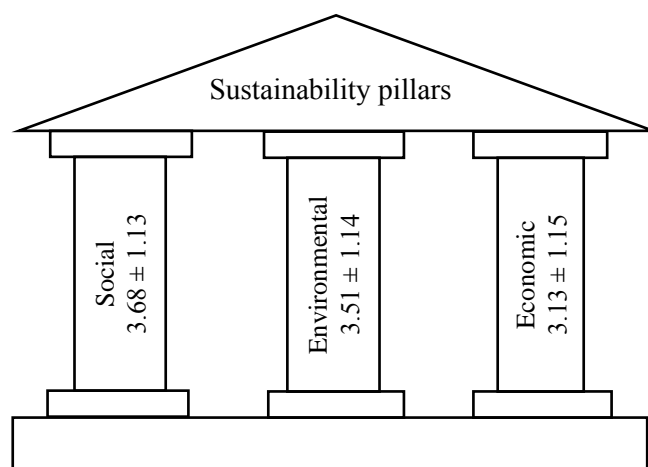
Demographic characteristics show that females were the prevailing respondents (305 respondents, 61.7%), slightly above half of the respondents were below 35 years of age (258 respondents, 52.2%) and the majority of respondents (274 respondents, 55.4%) reported having at least four household members [13].

### 3.2. Sustainable attitudes

In order to enlighten our knowledge of respondents' attitudes towards food waste sustainability, nine statements (three statements related to the environmental dimension, three related to the economic dimension and three associated with the social dimension) were extracted and further processed from the research on household food waste in Serbia [13].

The environmental dimension shows that respondents see food packaging as a bigger problem than food waste, they feel disturbed by the amount of food being wasted and the wasted resources needed for food production, but they have no opinion whether food waste is an environmental problem due to food's biodegradability. The social dimension comprised of statements that respondents have a bad conscience when they waste food, they feel guilty because some people do not have enough to eat, but have no opinion whether food they waste would help undernourished people. The economic dimension was associated with no opinion about the cost of food thrown away, regardless of whether respondents buy more than they plan during sales/promotions and whether they buy only from their shopping lists. A graphical presentation of summarized results for the three sustainable pillars is presented in Figure 1.





**Figure 1.** Sustainable attitudes towards food waste

Likert scale: (1) “Strongly disagree”, (2) “Disagree”, (3) “No opinion”, (4) “Agree”, (5) “Strongly agree

Table 1 shows amounts of self-reported total animal origin food waste/food packaging waste (N=494), per household and discarded in the last seven days. It can be observed that every second household discards more dairy products than meat or fish. It is assumed that half of the households discard this type of waste (dairy), and if they do, the amount is around 218.1 g (67.1 g per household member) or around 11.3 kg of food waste per year. Among 11 types of different food categories wasted in Serbia, quantities of discarded dairy products ranked fifth while fish and meat quantities were ranked ninth [13]. When it comes to food packaging waste, Table 1 shows that only 5% of respondents reported they did not discard food packaging waste. Speaking of numbers, below 30 pieces (around nine per household member) of different food packaging waste associated with animal origin food are discarded weekly. Innovative food packaging is recognized as an emerging future research perspective [19].

Taking into account the results from this study and the number of households in Belgrade and Serbia [20], we estimate that around 3,437 tons of animal origin food waste is annually discarded from Belgrade or 14,101 tons in Serbia. Regarding food packaging waste, the same calculations bring us to 3,723 tons of food packaging waste annually discarded from Belgrade or 15,275 tons in Serbia.

**Table 1.** Amount of self-reported animal origin food waste and food packaging waste in total (N=494), per household discarded in the last seven days (modified from [13])

Food category	Quantity per household, [gr] or [mL]	Number (%) of zero wasters
Dairy products	144.0 ± 364.1	265 (53.6%)
Fish/meat	74.1 ± 212.6	345 (69.8%)
Total amount of food waste per household	218.1 ± 482	234 (47.4%)
Food packaging category	Quantity per household [pieces]	Number (%) of zero wasters
Paper wrappers	12.7 ± 20.9	115 (23.3%)
Plastic wrappers	9.4 ± 18.8	168 (34.0%)
Tetra-pak packaging	6.7 ± 9.2	82 (16.6%)
Total amount of food packaging waste per household	28.8 ± 32.1	26 (5.3%)

Although meat and dairy products play an important role in human diets, they are associated with large amounts of greenhouse gas emissions during their life-cycle when compared to other foods of similar nutritive values [21, 22]. Based on the results from this study, further assumptions bring us to 1,763 tons of CO<sub>2</sub> emissions associated with annual animal origin food waste discarded in Belgrade and 7,234 tons in Serbia. When it comes to food packaging waste associated with this type of food, the CO<sub>2</sub> emissions are 172.2 tons in Belgrade and 706.4 tons in Serbia.

#### 4. Conclusion

This study builds on current knowledge of the overall environmental impact of animal origin food waste, as it is based on analysing reported data on food and food packaging waste in Serbia. Also, it provides a snapshot of respondents' attitudes towards the sustainability of food waste. Finally, this study shows that promotion of sustainable beliefs could help households improve their food management and decrease their quantities of animal origin food and food packaging.

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# Eating habits of Serbian consumers regarding content of fat and salt in meat products

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**Abstract:** In recent years, there is an increasing interest in developing meat products with healthier attributes. Consumers' behavior and expectations about sensory evaluation of traditional meat products is becoming less predictable. This paper reviews consumers' perception and acceptance of selected meat products from Serbia regarding content of fat and salt. Chicken and beef salami were sensory evaluated by consumers (n=3291) between 2014-2018. Sensory evaluation of the products was conducted according to a modified version of a DLG-5-points-scheme. The questionnaire started with general questions on demographic information about the respondents. Consumers rated the taste as good, satisfactory or unsatisfactory; the salt content as balanced, not salty enough or too salty; and the fat content as sufficient or too fatty. This investigation shows that Serbian consumers have become more interested in meat products with lower salt and fat contents. Consumers' trust and eating habits have changed over the years, and consequently, their acceptance of the products has also changed. Since consumer perceptions have a direct influence on meat industry profitability, the meat industry needs to know which attributes of meat products are considered important by consumers.

## 1. Introduction

Food and nutrition have been studied for centuries, focusing on dietary guidelines, general scientific advances, or particular nutritional therapies [1], but also were the subject of some political speeches. In the context of the stress and turmoil of the German revolution in 1848, the German philosopher Ludwig Feuerbach (1804-1872) used the phrase "We are what we eat" [2]. Martin Luther (1483-1546), German theologian, professor, pastor and church reformer, said "I eat and drink what I like and I will die when God wills it" [3]. Although food and nutrition have been analyzed from different points of views, modern nutritional science is surprisingly young. The first vitamin was isolated in 1926 and research on the role of nutrition in the human diet, and consequently, on human health (cardiovascular disease, diabetes, obesity, and cancers) is even more recent, accelerating over the past two or three decades and especially after 2000 [1].

A high consumption level of meat and meat products with significant amounts of saturated fat, sodium and sugar could exceed nutritional needs and consequently contribute to high rates of chronic diseases [4]. The Working Group of the International Agency for Research on Cancer (IARC) published a summary of their findings, in which they concluded there is an association of cancer with consumption of red meat or processed meat [5]. It was concluded that red meat can be linked to human colorectal cancer, and they assigned red meat to Group 2A "probably carcinogenic to humans". Most



developed countries are confronted with rising rates of diseases related to unhealthy eating habits, particularly the excessive consumption of salt, saturated fat and free sugars. In 2004, WHO adopted its global strategy on diet, physical activity and health as part of the global strategy to reduce chronic diseases [6]. The food industry was encouraged to limit and reduce the levels of *trans* fatty acids (FA) and saturated FA, salt, and free sugars in existing foods that are acceptable to consumers. The scientific EU projects such as PLEASURE [7] and TeRiFiQ [8] are helping industry to develop innovative processes and/or implement novel technologies to allow for the development and production of food products with low contents of harmful compounds (saturated and *trans* fatty acids, salt, and sugar).

The salt, fat and sugar contents of meat and meat products influence their structure, safety and nutritional quality. Salt ensures microbiological safety and affects biochemical reactions. On the other hand, fat in meat products is considered a valuable source of energy, fatty acids and vitamins. A TeRiFiQ study [8] showed that healthier dry sausages (with reduced salt and fat contents) can be manufactured with no adverse effect on the end-products' physical-chemical and biochemical properties. However, reducing or substituting the salt, fat and sugar content of a meat product could affect some of its sensory properties [9]. Hence, sensory studies are necessary to investigate final texture properties and consumer's acceptability of new, healthier meat products.

Smoked meat and meat product production and consumption in Serbia has been handed down over centuries, during which time, the tradition developed such that it has become a trademark. Although, these products are a significant part of the human diet in Serbia, in recent years, consumers in general have become more aware of the advantages of a healthy diet [9-11]. Consumers' perception and acceptance of meat products are critical issues for the meat industry. Thus, 'healthier' meat could offer benefits for both public health and the meat industry, but only if such products are accepted by consumers. The objective of this study was to evaluate the eating habits of Serbian consumers regarding the content of fat and salt in different traditional meat products over time (2014-2018).

## 2. Materials and Methods

Sensory evaluations of selected traditional smoked meat products from Serbia were carried out in 2014, 2016, 2017 and 2018 year in large retail stores in Belgrade. Consumers ( $n_{2014} = 85$ ;  $n_{2016} = 1157$ ;  $n_{2017} = 1018$ ;  $n_{2018} = 1031$ ) were males and females older than 18 years of age. The questionnaire started with general questions about the consumers, referring to their age, number of family members, education levels and shopping habits. Thereafter, respondents were asked to evaluate different traditional smoked meat products concerning their taste, color, salt and fat content, smoke flavor, etc. The origin and market name of the products were unknown to the consumers. Production of the analyzed meat products has been standard for the study years (2014-2018); composition (raw material, meat, fat tissue, salt, species and additives) was the same each year. Sensory evaluation of the products was conducted according to a modified version of an examination scheme developed by professional panels of scientists and practitioners in Germany (DLG-5-points-scheme). DLG test (*Deutsche Landwirtschafts-Gesellschaft*, German Agricultural Society) [12] is a descriptive sensory analysis which included visual (appearance/exterior), haptic (consistence/texture), olfactory (odor) and gustative (taste) criteria of the meat products.

## 3. Results and Discussion

Some results of sensory evaluation as well as descriptions of the analyzed meat products were described in detail in our previous publications [13-18]. This paper evaluates changes of consumers' attitudes towards taste, salt and fat content of the same meat products during four years of sensory evaluation. Consumers rated the taste as good, satisfactory or unsatisfactory; the salt content as balanced, not salty enough or too salty; and the fat content as sufficient or too fatty. Extracted results of sensory evaluations of different meat products are shown in Tables 1-2.

Table 1 shows sensory evaluation of taste and salt content for four types of salami as well as chicken cajna sausage in 2014 and 2016. Noticeably, chicken, beef and budim salami were evaluated

as too salty by a higher percentage of consumers in 2016 than in 2014. However, only the taste of budim salami and chicken cajna sausage was evaluated as good by a lower percentage of people in 2016 than in 2014. Consumers were more satisfied with both taste and salt content of homemade salami in 2016 than in 2014.

**Table 1.** Consumers' attitudes (% of consumers) in relation to salt content (*too salty*) and taste (*good*) of five meat products over time, n=1242.

Year	Answers	Chicken salami	Beef salami	Homemade salami	Budim salami	Chicken cajna sausage
2014	<i>too salty</i>	6.0	2.4	14.1	9.5	7.1
	<i>taste - good</i>	69.1	55.3	62.4	63.5	69.1
2016	<i>too salty</i>	15.0	7.0	7.5	10.5	5.0
	<i>taste - good</i>	77.0	70.0	66.0	48.5	54.0

Chicken, beef and royal salami were evaluated for taste, salt and fat content during 2017 and 2018 (Table 2). Sensory evaluation of all three types of salami showed that consumers' acceptance of the analyzed products was significantly lower in 2018 than in 2017. Consumers were more dissatisfied with the salt and fat content as well as with the taste. For the analyzed salami, the salt content was evaluated as too salty by almost two and a half to four and a half fold greater percentages of consumers in 2018 than in 2017. Similarly, the fat content of beef salami was evaluated as too fatty by as much as a six fold greater percentage of consumers in 2018 than in 2017. Consequently, consumers evaluated the taste as good in 2018, a significantly lower percentage in comparison with a year before. Our earlier research [13] indicated that traditional smoked meat products from Serbia, from the DLG experts' point of view, would enrich the West European market. High salt content and a too strong smoky flavor of the meat products were the main deficiencies. However, this investigation shows that Serbian consumers have become interested in foods with lower salt as well as fat content.

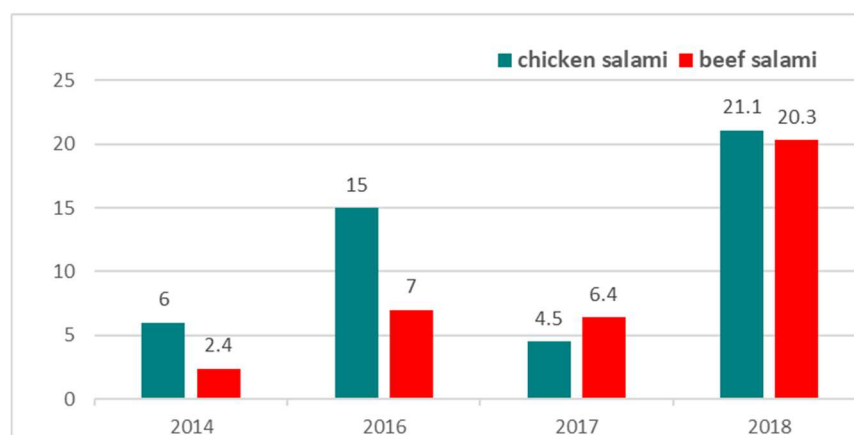
**Table 2.** Consumers' attitudes (%) in relation to salt content (*too salty*), fat content (*too fatty*), and taste (*good*) of chicken, beef and royal salami over time, n=2049.

	Answers	Chicken salami	Beef salami	Royal salami
2017	<i>too salty</i>	4.5	6.4	7.4
	<i>too fatty</i>	10.6	3.7	16.5
	<i>taste - good</i>	84.4	85.1	88.6
2018	<i>too salty</i>	21.1	20.3	17.5
	<i>too fatty</i>	24.9	23.1	31.1
	<i>taste - good</i>	57.8	71.5	66.3

WHO Member States have agreed to reduce the global population's intake of salt by a relative 30% by 2025 [19]. Reducing salt intake has been identified as one of the most cost-effective measures countries can take to improve population health outcomes. For example, the German Federal Ministry of Food and Agriculture recently presented a national reduction and innovation strategy for sugar, fats and salt in finished products as well as the National Action Plan for the prevention of poor dietary habits, lack of physical activity, overweight and related diseases [20]. Meat products with reduced fat levels, reduced sodium and nitrite contents, and enriched with functional ingredients are currently receiving much interest in the food industry and are well documented in scientific studies [21-23].

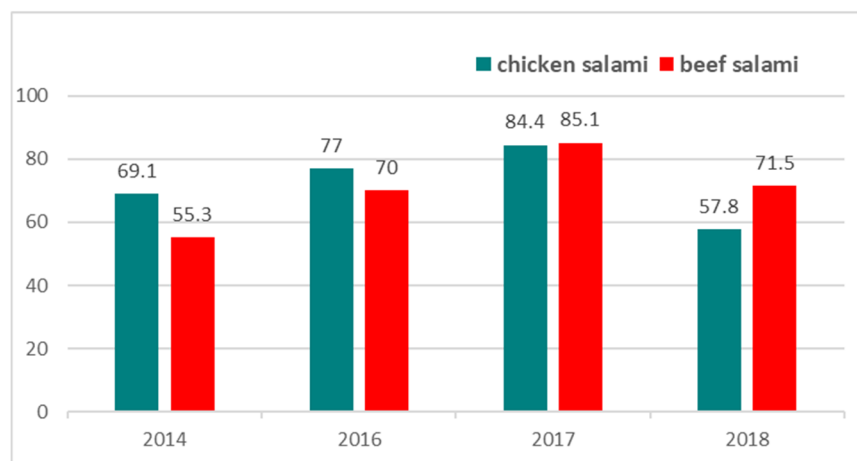
Proximate compositions of different smoked meat products from Serbia were analyzed in a few common studies [24-26]. The salt content of the analyzed meat products ranged between 3 and 6.1%. According to the literature data, the average salt content for these products is 4.5% [27]. In this paper,

the changes in consumers' attitudes to the salt content and taste of chicken and budim salami, production of which has been standard for years (2014-2018), is noticeable (Figures 1-2).



**Figure 1.** Consumers' attitudes (% of consumers) in relation to the salt content (*too salty*) over time, n=3291.

Sensory evaluation showed that consumers had different attitudes in the relation to the salt content over time (Figure 1). The highest percentages of consumers were dissatisfied with the salt content of chicken and beef salami in 2018 (*too salty*: 21.1% and 20.3%, respectively). It should be noted that during all years of testing, the demographic characteristics of consumers (gender, age, education level and number of household members) was included in the questionnaires and statistical evaluation of the data obtained will be the subject of the next publication.



**Figure 2.** Consumers' attitudes (% of consumers) in relation to the taste (*good*) over time, n=3291.

Changes in consumers' attitudes towards the salt content had influence on the consumers' taste assessment of the products (Figure 2). The taste of chicken and beef salami was evaluated as good by the lowest percentage of consumers in 2018 (57.8%) and in 2014 (55.3%), respectively.

The changes in Serbian consumers' perception should be taken into account, as well as the main deficiencies, related to the meat products' consistency, odor, and taste, as established by DLG experts [14]. Moreover, between 2009 and 2018, traditional meat products from Serbia passed the DLG tests

and receive “DLG award winner” medals [14,17], even with the mentioned established deficiencies, which could be very helpful for meat producers.

#### 4. Conclusion

This investigation shows that Serbian consumers have become more aware of the advantages of healthy foods with lower salt as well as fat contents, due to obvious advances in comprehending the relationship between diet and health. Consumers’ trust and eating habits have changed over time, and consequently, consumer acceptance of the products has also changed. Since the consumer perception of meat products has a direct influence on meat industry profitability, the meat industry should satisfy consumers’ needs and develop products that will be more acceptable to them.

#### Acknowledgement

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# Fatty acid profile of milk

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**Abstract.** Quality, processing ability and sensory properties of milk are highly correlated with content and composition of milk fat. Biologically active lipid substances are primarily saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs; linoleic acid; C18:2 n-6) and polyunsaturated fatty acids (PUFAs;  $\alpha$ -linolenic acid; C18:3 n-3). PUFAs with 20C, mainly docosahexaenoic acid (DHA; C20:5 n-3) and eicosapentaenoic acid (EPA; C22:6 n-3), are precursors of eicosanoids, which regulate various physiological processes. Fatty acid composition depends on many different factors, such as animal species, breed, season, lactation stage, geographical location, and diet. Goat and sheep milk are rich in the medium chain fatty acids, caproic (C6:0), caprylic (C8:0) and capric (C10:0), which is the reason for the specific aroma of those kinds of milk. Goat and sheep milk have more conjugated linoleic acid, and usually lower n-6/n-3 ratios, with higher amounts of  $\alpha$ -linolenic acid, compared to cow milk. Compared to goat and cow milk, sheep milk has the lowest amounts of lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acids i.e. fatty acids associated with negative effects on human health. The addition of forage, especially fresh grass, to dairy animal diets enhances the proportion of unsaturated fatty acids in milk fat compared to SFAs and increases the amount of conjugated linoleic acid.

## 1. Milk fat and fatty acid profile

Nutritional quality, processing ability, taste and aroma of milk and dairy products are highly correlated with content and composition of milk fat. Cow milk contains from 3.3 to 4.4% milk fat, goat milk from 3.25 to 4.2% and sheep milk contains approximately 7.1% milk fat. Milk fat contains a complex mixture of various lipid substances. These lipids are primarily triglycerides (triacylglycerides) which, by weight make up 98% of the total milk fat, while other milk lipids are diacylglycerides (0.25-0.48%), monoacylglycerides (0.02-0.04%), phospholipids (0.6-1.0%), cholesterol (0.2-0.4%), glycolipids (0.006%) and free fatty acids (0.1-0.4%). Over 400 different fatty acids, with unique physico-chemical and biological properties, constitute the triglycerides in milk [1]. Biologically active lipid substances are primarily monounsaturated fatty acids (MUFAs), oleic acid (C18:1 n-9), polyunsaturated fatty acids (PUFAs), linoleic acid (LA; C18:2 n-6) and  $\alpha$ -linolenic acid (ALA; C18:3 n-3). PUFAs with 20C, mainly docosahexaenoic acid (DHA; C20:5 n-3) and eicosapentaenoic acid (EPA; C22:6 n-3), are precursors of eicosanoids, which regulate various physiological processes [2]. From the aspect of human health, consumption of SFAs, mainly lauric, myristic and palmitic (C12:0, C14:0, C16:0, respectively) fatty acids, is associated with increased concentrations of low density lipoprotein (LDL) in blood, while other SFA from milk neutralise their effect because they increase high density lipoproteins (HDL) in blood. On the contrary, unsaturated fatty acids are regarded as beneficial for human health. DHA constitutes the main structural component of the brain cinerea, retina, and semen. It also participates in development of the nervous system, in the vision process, in development of premature babies and children, and has a role in prevention of inflammation [3, 4, 5, 6]. Historical facts indicate that the n-6/n-3 fatty acid ratio 1:1 was an important factor for human evolution. Global replacement of saturated animal fats with unsaturated plant fats, as well as intensive milk and meat production, primarily based on intensive grain fattening of animals, has led humans to



increased intake of linoleic acid (LA), precursor of the n-6 PUFA group. Since the n-6 PUFA group are competitors with the n-3 PUFA group for desaturation enzymes, the ratio of n-6/ n-3 fatty acids in the diet of today's humans has been altered from 1:1 to 10-20:1, or even higher, which can partly explain the rise of modern diseases, such as cardiovascular disease, cancer, obesity and diabetes [7]. The n-3 fatty acids are associated with improved neurological functions, coronary heart disease protection, and anti-carcinogenic effects [8]. Both n-groups of PUFA are constituents of cell membrane phospholipids, where they have a role in maintaining the functionality of the membrane [9].

The concept of functional dairy products has recently gained attention due to the positive effects of conjugated linoleic acid (CLA), long chain PUFA, especially the two isomers *cis*-9, *trans*-11 (rumenic acid) and *trans*-10, *cis*-12, on human health. In ruminants, CLA is produced naturally from dietary LA, ALA and *trans* vaccenic acid. CLA is synthesised in the rumen during ruminal biohydrogenation of dietary fatty acids, or in tissues by  $\Delta$ -9 desaturase enzyme activity. *Trans* vaccenic acid provides the substrate for endogenous synthesis of CLA through the activity of  $\Delta$ -9 desaturase, especially in the mammary gland and other body tissues [10]. Rumenic acid, which makes 75-90% of total CLA content in milk fat, is the most active biological natural isomer of CLA in dairy products [11]. CLA protects against cancers in various experimental animal models and human cells. Also, CLA has anti-obesity and anti-atherogenic effects in humans and animals [12, 13, 14, 15].

## 2. Variation in milk fatty acids content

Given the fact that the cow milk fatty acid profile depends on the fatty acids originating from feed and the biohydrogenation process that occurs in the rumen, the fatty acid composition depends on many different factors such as breed, season, lactation stage, lactation number, age of dairy cows, geographical location, and, as most important factor, the diet, which is responsible for 95% of the variance in cow milk fat [8, 16, 17, 18]. Lock and Garnsworthy [19] showed seasonal variability in the CLA content, measured *via*  $\Delta$ 9-desaturase activity, while Elgersma et al. [20] noted seasonal changes in cow milk CLA content between winter and summer. Peterson et al. [21] showed individual animal differences in  $\Delta$ 9-desaturase activity, which is an indirect indicator of the variation in fat content. The seasonal changes of the feed ratio between grass silage and fresh herbage influence the fatty acids, meaning increased amounts of long chain fatty acids (C17-24), increased unsaturated/saturated fatty acid ratio, and decreased amounts of medium chain fatty acids (C12-C16) [4]. The average content of rumenic acid in milk of pasture-fed cows is two to three times higher than in barn-fed cows [11]. Soyeurt et al. [22] showed differences in the fatty acid content of milk across the studied dairy breeds, which suggest milk and dairy products with improved fatty acid composition could result from choosing the right breed. However, variations within breeds were also found [22].

Beside these variations, there are differences between fatty acid profiles of milk from different animal species. Cow milk fat contains on average 60-70% SFA. The main SFA in most mammals' milk fat is palmitic acid (C16:0). The fat of goat and sheep milks is rich in the medium chain fatty acids, caproic, caprylic and mostly, capric fatty acid (C6:0, C8:0, C10:0, respectively). Sheep milk, compared to goat and cow milk, had the lowest amount of lauric, myristic and palmitic acids (C12:0, C14:0, C16:0, respectively), associated with negative effects on human health. A characteristic of goat milk is a lauric/capric acid ratio <0.5, while this ratio in cow and sheep milk is >1. This parameter can be important indicator for detection of milk falsification. The higher concentrations of caproic, caprylic and capric fatty acids in goat and sheep milks than in cow milk is the reason for the specific aroma of goat and sheep milks. Goat and sheep milks have more CLA than cow milk, probably because small ruminant breeding practices are usually semi-extensive, and consequently, their diet is more rich in forage [6].

The amount of MUFA is similar among cow, goat and sheep milks, and it ranges from 20% to 35% of the milk fat. Oleic acid is the most abundant fatty acid from this group. In cow milk, there is about 24%, while in goat and sheep milks, there is about 18% oleic acid. Goat and sheep milks usually have lower n-6/n-3 ratios, and higher amounts of ALA than cow milk [6, 22].

**Table 1.** Fatty acid composition in milk of three dairy animal species (molar percentage) [23]

Fatty acid	Cow	Sheep	Goat
Short chain			
C4:0	11	8	8
C6:0	5	5	5
C8:0	1	4	4
Medium chain			
C10:0	3	6	13
C12:0	3	5	7
C14:0	10	10	12
Long chain			
C16:0	23	22	24
C18:0	10	10	12
C18:1	29	22	17
C18:2	2	4	3
C18:3	<1	<1	<1

\*Common fatty acids of cow, sheep and goat milk are C16:0, C18:0, C18:1, C18:2 and C18:3

### 3. The influence of feed on the milk fatty acid composition

One of the strategies to prevent chronic non-infectious disease development in humans is to change the fatty acid composition of their diets. This strategy is based on reduced intake of SFA, increased intake of MUFA and PUFA, and especially, the change of the n-6/n-3 fatty acid ratio, while maintaining the sensory properties of milk and milk products.

Interest in milk's fatty acid composition and the opportunity to change it with dietary/feed modifications, began in the 1980s, when some authors published studies about the use of linseed oil in cow diets and the effect on milk fat composition. However, until the appearance of gas-liquid chromatography, such studies were rare [24]. Nowadays, the composition of milk fatty acids is of great interest with respect to human nutrition, as alteration of fatty acids in cow diets can influence human health [25].

The diet of dairy cows, which affects the microbiological processes in rumen, and the changes in the biohydrogenation processes, are the key to modifying the fatty acid composition of milk fat [26, 27]. Lipid metabolism, specifically ruminal biohydrogenation, is influenced by factors such as rumen pH, and the amount, source and fatty acid profile of the fat supplements in the animal diets [28].

Still, in spite of data confirming that milk fatty acids are susceptible to changes depending on the animal diet, the results often cannot be compared, due to the large differences in feed composition. While short chain (4 to 8 carbons) and medium chain fatty acids (10 to 14 carbons) are synthesized *de novo*, very few long chain fatty acids can be synthesized *de novo* by ruminants; instead, these fatty acids must be ingested with the feed [20, 27, 28]. Milk fat content generally increased with increasing fibre content of different forage [27, 29, 30]. The addition of forage, especially fresh grass, enhances the proportion of unsaturated fatty acids in cow milk fat compared to saturated fatty acids [20, 31]. Similarly, Chilliard et al. [28] reported that the content of PUFA, especially C18:3, C18:0 and C18:1, and SFA, especially C16:0, can be changed if the content of the hay, fresh grass and maize silage in the diet is increased. Also, the intake of fresh grass increases the concentration of CLA, the major 9-*cis*, 11-*trans* isomer, a biologically active compound with anti-carcinogenic and other beneficial effects on human health [12, 32]. Similar to this, other authors suggested that when fat supplements are added to diet, the response in milk production and composition is more variable than when diets are totally or mostly based on corn silage as forage [26, 27]. On the other hand, diets with higher concentrate contents can provide large amounts of digestible carbohydrates, and reduced amounts of fibrous components, which can cause milk fat depression and change of the milk fatty acid profile [27, 30]. A dairy cow diet containing added linseed oil, which is rich in ALA, increases PUFA in milk,

especially ALA and *cis*-9, *trans*-11 CLA [33], while addition of sunflower and fish oil increases vaccenic acid and *cis*-9, *trans*-11 CLA [34]. If the ruminal biohydrogenation process is complete, unsaturated fatty acids are transformed to SFA, which is main cause of concern for human health. But if this rumen process is controlled and unsaturated fatty acids transform to stearic acid, it should be possible to improve the healthiness of cow milk, through increased amounts of CLA and n-3 fatty acids [35]. Fish oil, containing EPA and DHA, inhibits the complete biohydrogenation of C18 unsaturated fatty acid, which increases the *trans* 18:1 isomer available for synthesis of CLA isomers [36].

#### 4. Conclusion

Differences in the fatty acid content in milk across the species, breed and season, or differences based on diet, provides us with the ability to choose the right breed, diet or breeding conditions to obtain milk and dairy products with improved nutritional quality and more valuable fat composition. Consuming milk with a more valuable composition should positively influence consumer health.

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# The nitrite content in domestic and foreign cooked sausages from the Serbian market

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**Abstract.** The nitrite content (expressed as NaNO<sub>2</sub>) was measured in 236 samples of different types of domestic and foreign cooked sausages from the Serbian market, according to standard ISO procedure. The highest content of nitrite (expressed as NaNO<sub>2</sub>) was found in fine comminuted cooked sausages from foreign producers. Similarly, the coarse comminuted cooked sausages from foreign producers contained higher nitrite compared to domestically produced cooked sausages. According to National and EU Regulations, the maximum allowed nitrate content (expressed as NaNO<sub>2</sub>) is 150 mg/kg. All tested cooked sausages had nitrite levels below the regulatory limits set by National Regulation and Regulation (EC) No. 601/2014, but the highest nitrite contents were found in cooked sausages from foreign producers.

## 1. Introduction

Nitrites are one of the most important additives in the meat industry because of their beneficial effect on the quality and microbiological safety in meat products. Nitrite has been used for preservation of meat products (sausages, ham, bacon) and is an efficient inhibitor of the growth of pathogenic bacteria (*Clostridium botulinum*, *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium perfringens*) [1-2] and, thereby, decreases the risk of *Clostridium botulinum* producing toxins [3]. However, nitrite provides the processed meat with its sensory characteristic (color and aroma) and it inhibits lipid oxidation processes [4]. On the other hand, the major concern of nitrites in meat products is related to the potential of nitrites to form carcinogenic compounds. Over the past several decades, the nitrite content in different meat products was evaluated [5-9]. Nitrite contents of foodstuffs should be monitored in the context of dietary intake and to provide insights into new manufacturing meat technologies.

The aim of this study was to determine the nitrite content in cooked sausages (fine and coarse) from domestic and foreign producers in the Serbian market.

## 2. Materials and Methods

A total of 236 samples of different types of cooked sausages (fine and coarse comminuted cooked sausages) were obtained from different producers (domestic and foreign); sausages were available on the Serbian market. The nitrite content (mg/kg) was determined according to standard ISO procedure [10] and expressed as NaNO<sub>2</sub>. The cooked sausages were sampled and homogenized in a Braun





CombiMax 600 homogenizer (Braun, Germany) and analyzed immediately. The nitrite content of different type of cooked sausages was evaluated according to Regulation (EC) No. 601/2014 (Table 1).

**Table 1.** Nitrite content limits reported in Regulation (EC) No. 601/2014

Meat products	Nitrite legal limit (mg/kg)	Limit application
Non-heat- treated processed meat	150	Maximum amount that may be added during manufacturing.
Heat-treated processed meat	150	Maximum amount that may be added during manufacturing.
Only sterilized meat products ( $F_0 > 3.00$ )*	100	Maximum amount that may be added during manufacturing.
Traditionally cured meat products with specific provisions concerning nitrites and nitrates	50-180	Depends on product: Maximum added amount or maximum residual amount, residue level at the end the production process.

\*  $F_0$ -value 3 is equivalent to 3 minutes heating at 121 °C for *C. botulinum*

The statistical analysis of the results was performed using the GraphPad Prism version 7.00 software. The nitrite content of cooked sausages from the Serbian market were expressed as the mean  $\pm$  standard deviation and were subjected to analysis of variance (One-way ANOVA). The graphical presentation of nitrite content was performed using Microsoft Office Excel 2010.

### 3. Results and Discussion

The nitrite contents (expressed as  $\text{NaNO}_2$ ) of the cooked sausages from domestic and foreign producers are shown in Table 2. The nitrite content in fine comminuted cooked sausages from domestic producers ranged between 3.13 mg/kg and 76.42 mg/kg. The mean content of nitrite in fine comminuted cooked sausages from foreign producers ranged between 6.42 mg/kg and 62.09 mg/kg. Similarly, mean content of nitrite in coarse comminuted cooked sausages from domestic producers ranged between 3.15 mg/kg and 33.97 mg/kg. The mean nitrite content in coarse comminuted cooked sausages from foreign producers ranged between 8.31 mg/kg and 58.73 mg/kg.

**Table 2.** Nitrite content (expressed as  $\text{NaNO}_2$ ) in cooked sausages from the Serbian market (mg/kg)

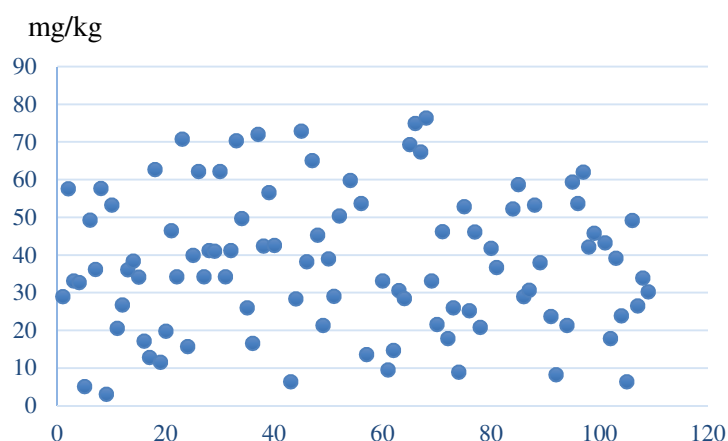
Statistics	Type of sausages			
	Fine comminuted cooked sausages		Coarse comminuted cooked sausages	
	Producers		Producers	
	Domestic	Foreign	Domestic	Foreign
N*	136	32	56	12
Mean $\pm$ SD	38.97 $\pm$ 19.44	39.14 $\pm$ 17.15	22.46 $\pm$ 16.83	34.26 $\pm$ 15.80

Standard error of Mean	2.37	4.95	9.71	3.95
Range (min-max)	3.13-76.42	6.42-62.09	3.15-33.97	8.31-58.73

\*N – number of examined samples.

Reinik et al. [11] found the mean sodium nitrite contents in ham were 20.8 mg/kg. However, in our current study, the mean nitrite concentration in cooked sausages ranged between  $22.46 \pm 2.62$  mg/kg and  $39.14 \pm 17.15$  mg/kg. Öztekin et al. [12] presented the nitrite content in ham; 35.6 mg/kg. Stalikas et al. [13] reported that nitrite content in salami were 54 mg/kg. Thus, differences are more likely to be due to the manufacturing processes and different meat products. It was reported by Dennis et al. [9] that the mean nitrite content in bacon was 24.0 mg/kg. Siu and Henshall [14] reported the nitrite content in salami was 108.0 mg/kg.

The distribution of the nitrite contents is presented in Figure 1. The highest nitrite content was detected in fine and coarse comminuted cooked sausages from foreign producers, means of  $39.14 \pm 17.15$  mg/kg and  $34.26 \pm 15.80$ , respectively. According to the Commission Regulation [15] and the National Regulation [16] nitrite content must be lower than 150 mg/kg. All tested cooked sausages met this regulation.



**Figure 1.** Distribution of nitrite content (expressed as  $\text{NaNO}_2$ ) in cooked sausages from the Serbian market

Different countries have set their maximum limits for the addition of nitrite salts in meat products [7]. Under the Serbian National Regulation [16] the maximum nitrite (potassium or sodium salts) permitted is 150 mg/kg. Given the established antimicrobial effect of nitrite salts, its level should remain sufficient to prevent the growth of foodborne hazards. On the other hand, it is advisable to minimize dietary nitrite intake in light of nitrite's potential adverse health effects. Nitrite contents in the various cooked sausages were below the maximum allowable limit set by National Regulation [16] and Commission Regulation [15], but the actual amount of nitrite was higher in sausages from foreign than domestic producers.

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## Anti-genotoxic potential of *Gentiana lutea* extracts against the food sweetener saccharin

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**Abstract:** Sweeteners are now an integral part of the food industry. Despite its long history of use, saccharin has been in the spotlight for many reasons including toxicity, cancerogenicity and other health issues. Because of this, it is necessary to find natural supplements that could reduce the negative effect of this sweetener. The aim of the study was to investigate the potential anti-genotoxic effect of *Gentiana lutea* plant extracts against saccharin-induced genotoxicity. *G. lutea* is widely used in traditional medicine and in the food and pharmaceutical industries. The anti-genotoxicity of root and leaf 50% aqueous-ethanolic extracts was tested on normal human fetal fibroblasts (MRC-5) and human hepatocarcinoma cells (HepG2) using the comet assay. *G. lutea* extracts significantly reduced the genotoxic effect of saccharin at all tested concentrations. The results obtained in this study indicate the strong protective effect of *G. lutea* against saccharin-induced DNA damage and encourage further studies in order to use this plant as a source of natural food supplements.

### 1. Introduction

Food additives are used in the food industry to improve food quality, safety, flavor, sweetness and other properties of food [1]. In order to determine the relationship between diet and cancer, it is necessary to determine which food chemicals are potential mutagens. Most chemicals are present in food in relatively low concentrations; however, some of them possess carcinogenic potential even at very low concentrations, and so it is important to reduce or eliminate their harmful effect. It is known that extensive sugar consumption has harmful effects on human health, and moreover, its use by diabetics is limited. To overcome this problem, artificial sweeteners started to appear in the 1800s. One of the most common is saccharin, discovered in 1879 [2]. Since its discovery, saccharin has been the center of many controversies regarding its potential toxic effects. It is the most intensively investigated sweetener for its possible carcinogenic effects.

In order to decrease side effects of artificial food additives, there is a constant need to develop natural products that are able to substitute artificial additives, or to attenuate their harmful potential. Numerous available drugs originating from plant material are widely used in traditional medicine. One of them is manufactured from *Gentiana lutea* or great yellow gentian, a medicinal plant that possesses various biological activities. Gentian was considered successful in the treatment of liver and stomach problems, fever, infected wounds, and is still used in folk medicine to improve appetite and stimulate



digestion. Also, it has anti-inflammatory, antioxidant, hepato-protective, diuretic, and antidiabetic effects [3, 4]. Gentian root has a long history of use because of its bitterness, and it is the main ingredient of many pharmaceutical products.

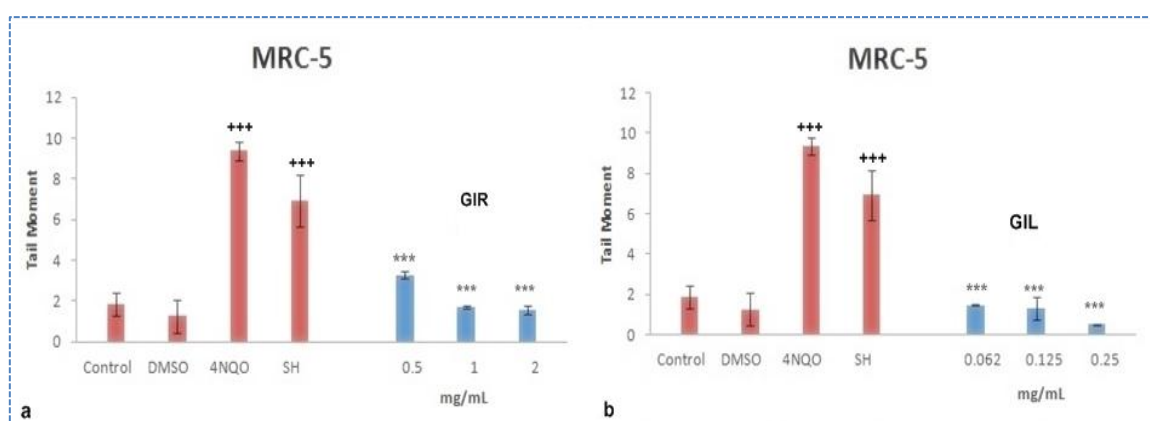
Accordingly, the aim of this study was to examine the anti-genotoxic potential of *G. lutea* extracts towards the genotoxic effect of saccharin.

## 2. Materials and Methods

Plant extracts were prepared as previously described by Nastasijević et al. (2012) [3]. Firstly, to determine non-cytotoxic concentrations of *G. lutea* root (GIR) and leaf (GIL) 50% aqueous-ethanolic extracts and saccharin (SH) towards normal human fetal fibroblasts (MRC-5) and human hepatocarcinoma (HepG2) cell lines, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Tested concentrations ranged from 0.062 mg/mL to 2 mg/mL for extracts and 0.312 mg/mL to 10 mg/mL for saccharin. MTT assay was performed as described by Vasilijević et al. (2018) [5], measuring the absorbance at 570nm to determine cell viability. To investigate genotoxic and anti-genotoxic potentials of extracts and sweetener, the alkaline comet assay was applied as previously described by Nikolić et al. (2015) [6]. The DNA damage was quantified by Comet Assay IV software. The highest non-genotoxic concentrations were selected for determination of anti-genotoxic effect of extracts. Both cell lines were exposed to co-treatment of extracts and saccharine for 24 h. For statistical analysis, Statistica 7.0 (StatSoft, Inc.) was used. To determine statistical significance, the Mann-Whitney *U* test was applied.

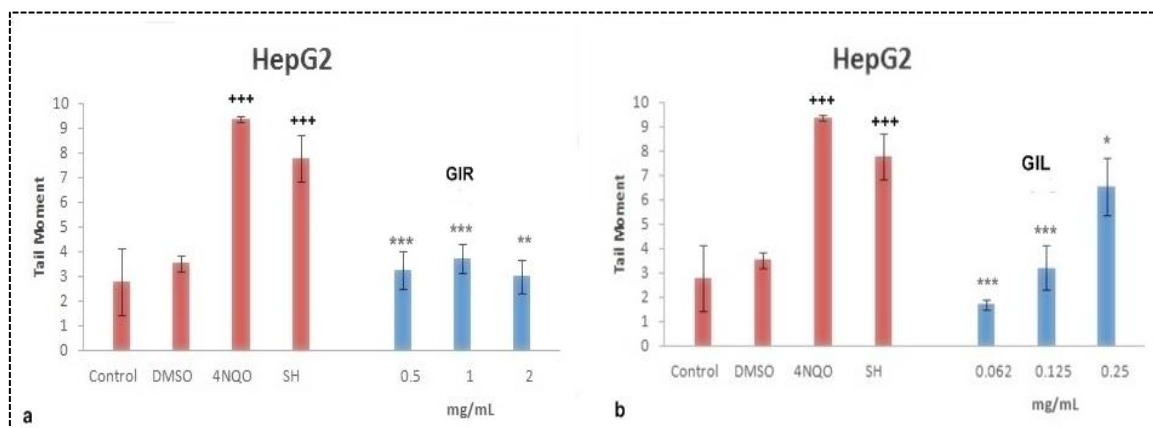
## 3. Results and Discussion

According to MTT results, all tested concentrations of *G. lutea* extracts (up to 2 mg/mL) did not show significant cytotoxicity towards MRC-5 or HepG2 cell lines, and were further used in genotoxicity/anti-genotoxicity studies. Furthermore, because the aim of the study was to examine the anti-genotoxic potential of *G. lutea* extracts, it was necessary to determine non-genotoxic concentrations of extracts and concentrations of saccharin that induced sufficient DNA damage. Using the comet assay (data not shown), concentrations ranging from 0.5 mg/mL to 2 mg/mL for root extract and 0.062 mg/mL to 0.25 mg/mL for leaf extract, and the saccharin concentration of 10 mg/mL, were chosen to test anti-genotoxic potential. Anti-toxicity was examined on both cell lines co-treated with genotoxic doses of saccharin (10 mg/mL) and serial concentrations of each plant extract for 24 h.



4-Nitroquinoline N-oxide (4NQO)-positive control (10 $\mu$ M); \*\*\* Significant differences between co-treated groups and saccharin; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001 +++ Significant differences in regard to dimethyl sulfoxide (control solution); + $p$ <0.05; ++ $p$ <0.01; +++ $p$ <0.001

**Figure 1.** Anti-genotoxic potential of *G. lutea* 50% ethanolic root (a) and leaf (b) extracts against saccharin on MRC-5 cell line. Results are presented as mean values of tail moment  $\pm$  SD



Results are presented as mean values of tail moment  $\pm$  SD; 4-Nitroquinoline N-oxide (4NQO)-positive control (10 $\mu$ M); \*\*\* Significant differences between co-treated groups and saccharin; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001 +++ Significant differences in regard to dimethyl sulfoxide (control solution); + $p$ <0.05; ++ $p$ <0.01; +++ $p$ <0.001

**Figure 2.** Anti-genotoxic potential of *G. lutea* 50% ethanolic root (a) and leaf (b) extracts against saccharin on HepG2 cell line

Results of testing the anti-genotoxic potential of *G. lutea* extracts against saccharin on the MRC-5 and HepG2 cell lines are shown in Figure 1 and Figure 2. The results indicated that all tested concentrations significantly reduced the genotoxicity induced by saccharin. Interestingly, the lower concentrations exhibited higher statistically significant anti-genotoxic effect, being in accordance with the dual features of some anti-genotoxic agents, termed *Janus* substances [7]. Bearing in mind that saccharin could induce oxidative stress/damage [8], the protective effect of *G. lutea* extracts could be attributed to their high antioxidative properties [3]. Our findings are in line with the work of Hudecova et al. (2010) [9] who showed that hydrogen peroxide-induced DNA damage could be significantly reduced with plants from *Gentiana* genus. Moreover, the radio-protective effect of *G. lutea* extracts was observed on peripheral blood mononuclear cells and human cervix carcinoma cells [10].

#### 4. Conclusion

The results obtained in this study showed that *G. lutea* ethanolic root and leaf extracts exhibited strong protective effects against DNA damage induced by saccharin. All tested concentrations of *G. lutea* extracts significantly reduce the genotoxicity of this sweetener. Taking into account the controversial nature of saccharin, data obtained in this study are a promising contribution to possibly reducing the genotoxicity of this sweetener. Further studies are needed in order to examine the potential of *G. lutea* extracts to be used as dietary supplements.

#### Acknowledgements

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# Effectiveness of using iodine-containing additives in meat products for child nutrition

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**Abstract.** Topical data on the prophylaxis of iodine deficiency conditions in children and adults are given. The results of a study on the effectiveness of using iodine-containing additives BioIod, iodcasein or iodized salt in meat products designed for childhood nutrition are presented. The use of iodine-containing additives BioIod or iodcasein in meat products for prophylaxis of iodine deficiency conditions in children's nutritional products was effective. With that, the highest effectiveness was observed for the BioIod additive, which was proved by experiments on hypothyroid model animals. Iodized salt has the least protective effect in iodine deficiency conditions.

## 1. Introduction

Iodine deficiency is the only and, according to the data of world statistics, the most common cause of brain damage and mental disorders that can be prevented [1,2]. Actively growing children and adolescents comprise a special risk group regarding the development of iodine deficiency diseases. Even slight nutritional deficiency of iodine reduces their intellectual abilities. There are different options of iodine prophylaxis. The most effective and economically advantageous option is mass iodine prophylaxis that consists of supplementation of food products with various iodine-containing additives. The biologically active additive iodcasein, intended for use in food products and known in the pharmacy chain as Iodactiv, was developed earlier and has become widespread. Iodcasein is an organic iodine compound incorporated into a milk protein molecule. It is used to treat iodine deficiency, and when administered in excess, it is excreted from the body while not entering the thyroid gland, since iodine splits from the milk protein by the action of the liver enzymes produced during iodine deficiency. When a person is iodine sufficient, these liver enzymes are not produced and iodine is naturally excreted from the body without absorption into the blood [3,4]. Recently, a new organic iodine additive has appeared on the market, i.e. BioIod, which is an analogue of natural iodotyrosine contained in animal and plant food products, the consumption of which the human body is evolutionally adapted to.

Nowadays, the diet of preschool and school-age children in the city of Moscow and other regions in Russia includes cooked sausages, ham products and minced meat semi-finished products for child nutrition manufactured according to legal national standards. Iodine supplementation amounting to 20-30% of children's daily physiological norm by using iodcasein is stipulated in these products [5,6].





## 2. Materials and methods

The mass fraction of iodine was determined in meat products for baby food by the inversion-voltammetric method. The child nutritional products, *Detskaya* cooked sausage and *Detskyi* schnitzel were prepared with BioIod, iodocasein or iodized salt added at appropriate levels. Schnitzel were cooked, and all cooked meat products were stored until the end of their shelf-lives. Iodine content in the sausages was examined before and after cooking and in the schnitzel before and after production of raw product. Then, iodine content of all cooked meat products was determined during storage.

A laboratory animal study consisted of two stages and lasted 50 days, during which time the rats consumed the diets prepared for rat groups 1-5 (below). Firstly, rats were prepared as a model of hypothyroidism (iodine deficiency) during days 1-25. Secondly, the drug Mercazolil was administered to rats daily intragastrically at a dose of 50 mg/kg body weight from day 26 to day 50 to stimulate hypothyroidism (iodine deficiency).

Rats were randomized by weight into five groups (control, intact and three experimental), with the components below included in their diets. Rats in groups 2-4 received diets enriched with iodine, providing 30 % of the animals' average daily requirement for iodine:

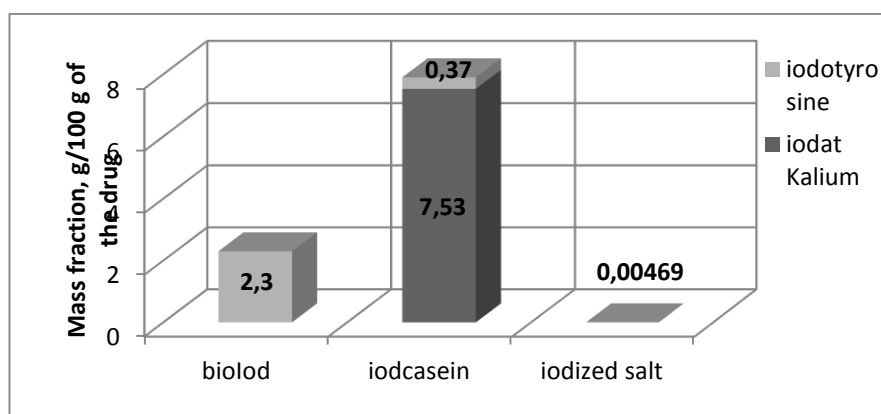
- 1) Control group – diet containing semi-finished products without iodine-containing additives;
- 2) Diet containing semi-finished meat product with iodocasein;
- 3) Diet containing semi-finished meat product with BioIod;
- 4) Diet containing semi-finished meat product with iodized salt;
- 5) Intact group – diet was the standard vivarium diet.

Rats were stunned then euthanized using CO<sub>2</sub> on day 25 (pre-hypothyroidism) or day 50 (hypothyroid). Blood was taken from the cardiac ventricles of stunned animals. Levels of three thyroid hormones, thyroxine (T4), triiodothyronine (T3) and thyroid-stimulating hormone (TSH) in blood sera of rats were measured using standard enzyme linked immunosorbent assays.

## 3. Results and discussion

The effectiveness of BioIod compared to iodocasein and iodized salt in sausages and ready-to-eat culinary meat products for child nutrition for prophylaxis of iodine deficiency diseases in children was studied.

In the first stage of the study, the amounts of iodine in the iodine-containing additives and iodized salt were determined so appropriate application rates for dietary enrichment could be calculated (Figure 1).

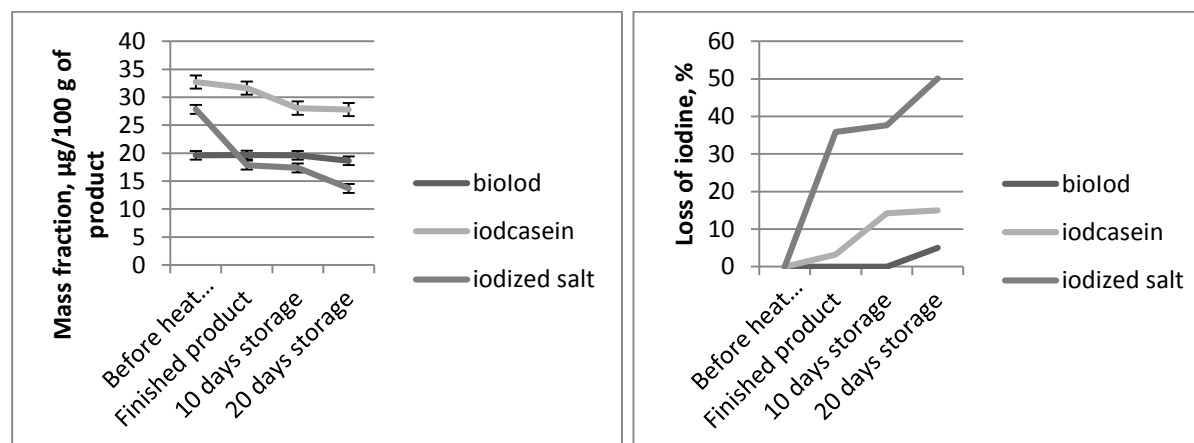


**Figure 1.** Iodine content in iodine-containing additives used in the study

In order to provide a consumer with a dose of 40 µg of iodine (when consuming 100 g of a product per day on the basis of the total amount of iodine in the additive), the additive application rate for *Detskaya* cooked sausage and *Detskyi* schnitzel, without regard for losses during cooking and storage, would be 1.3 g/100kg and 1.6 g/100 kg, respectively, for BioIod; 0.4 g/100kg and 0.5 g/100 kg, respectively, for iodocasein, and; 640 g/100kg and 500 g/100 kg, respectively, for iodized salt. In production of *Detskyi* schnitzel, however, the application of iodized salt does not ensure 30% of the recommended daily dose. To ensure the required amount of iodine, it would be necessary to add double

the amount of salt that is regulated in this product, so the use of iodized salt alone is unacceptable in this child nutritional product.

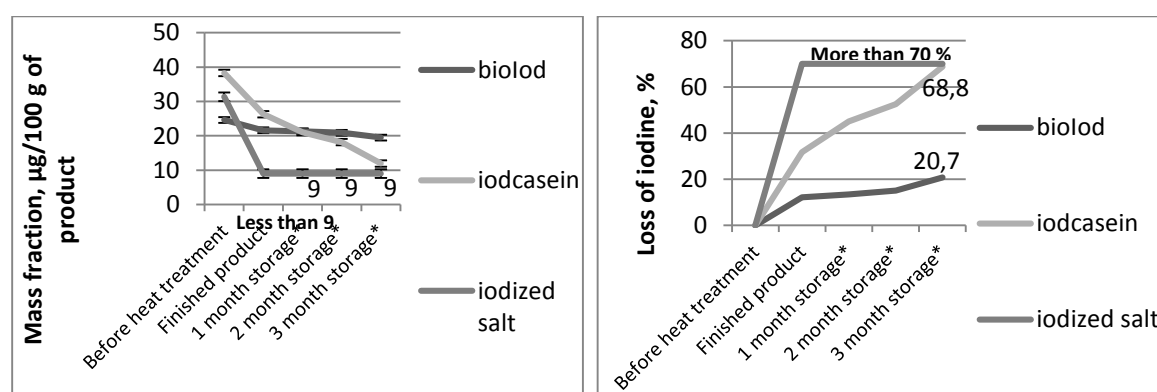
Iodine loss before and after cooking and during storage of *Detskaya* cooked sausages (Figure 2) and the meat product *Detskyi schnitzel* (Figure 3) was examined.



**Figure 2.** Changes in iodine content in *Detskaya* cooked sausage during storage

At the end of the shelf-life of *Detskaya* cooked sausages for child nutrition, iodine loss was 50% in iodized salt supplemented sausages, 15% in iodcasein supplemented sausages and 5% in BioIod supplemented sausages.

The BioIod application rate of 1.35 g/100 g of unsalted raw material, determined on the basis of the iodine content in BioIod, did not ensure an iodine content of 30 µg in the finished *Detskaya* cooked sausage. Also, iodine loss (5% of the initial content) occurred in *Detskaya* cooked sausage at the end of the shelf-life. Therefore, the actual required BioIod application rate would be 2.2 g/100 kg of unsalted raw material.



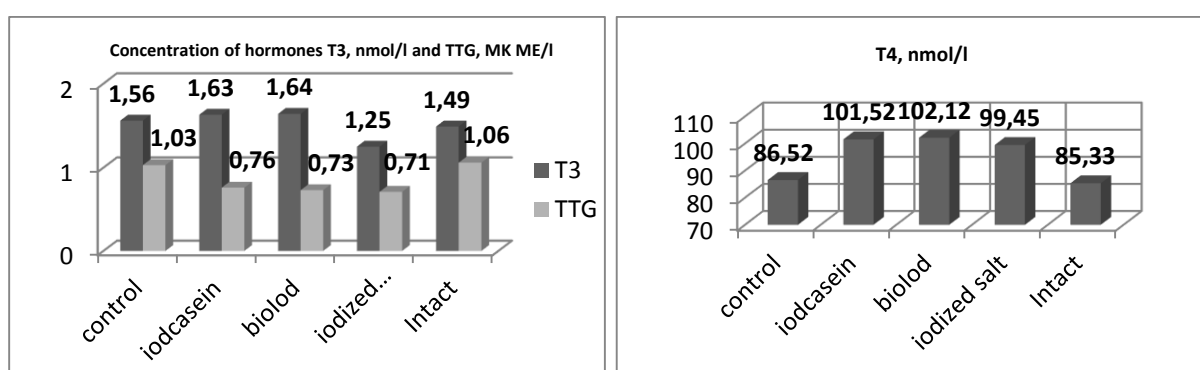
**Figure 3.** Changes in iodine content in *Detskyi* schnitzel during storage. \*Stored product underwent cooking at 180°C to a temperature of 90°C in the center

At the end of the shelf-life of *Detskyi* schnitzel for child nutrition, iodine loss was more 70% in iodized salt supplemented schnitzel, 68.8% in iodcasein supplemented schnitzel and 20.7% in BioIod supplemented schnitzel.

The BioIod application rate 1.7 g/100 g of unsalted raw material, determined on the basis of the iodine content in BioIod, did not ensure an iodine content of 40 µg in the finished *Detskyi* schnitzel. Also, iodine loss (20.7% of initial content) occurred in *Detskyi* schnitzel at the end of the shelf-life. Therefore, the actual required application rate of BioIod would be 3.5 g/100 kg of unsalted raw material.

The effectiveness of iodine-containing additives based on the milk protein casein or whey protein compared to iodized salt in the composition of meat minced semi-finished products for child nutrition was examined. The prophylaxis effect of meat products enriched with iodine-containing additives was studied using model, iodine-deficient laboratory rats on the basis of hormonal parameters. Pre-hypothyroidism (day 25) and final, hypothyroidic (day 50) blood hormone (T3, T4 and TTG) levels were measured in rats (Figures 4 and 5).

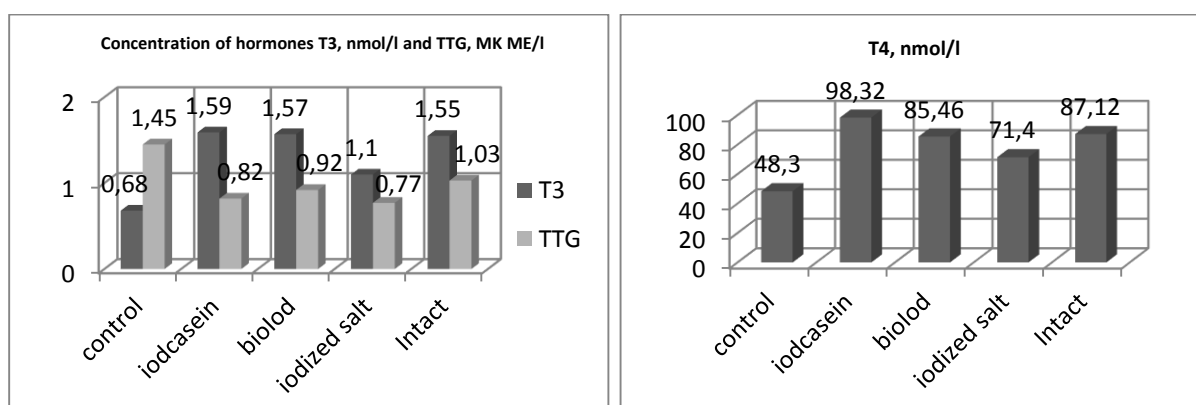
Analysis of animal hormonal status on day 25 showed serum T4 levels did not significantly differ between animals that did not consume iodine-enriched diets and animals that were injected with iodine-enriched products. At the same time, serum T3 levels in animals consuming diets enriched with milk or whey proteins (groups 2 and 3) were higher than in control and intact animals. T3 levels in group 4 animals that consumed meat products with iodized salt were lower than in control and intact animals. Lower serum TSH levels were found in all animals consuming iodine-enriched diets than in animals consuming the control diet and intact animals (Figure 4).



**Figure 4.** Thyroid hormone levels in the blood serum of animals before simulation of hypothyroidism (day 25)

Thus, the decrease in serum TSH levels in group 3 animals, and increases in serum T3 and T4 levels may indicate that BioIod-enriched meat products indirectly stimulated the production of thyroid hormones, which, in turn, likely changed the functional activity of the immune system and individual populations of immunocompetent cells, in particular, the differentiation of immature lymphoid cells. It should be stressed that in group 4 animals that consumed meat semi-finished products enriched with iodized salt, T3 levels increased (up to 17%), but T4 and TSH levels significantly decreased (reduction of up to 30%).

In the second stage of the model animal study (groups 1-4), clinical symptoms of developing hypothyroidism of different severity were observed – there was a decrease in motor activity (hypodynamia), an increase in goiter, detected by palpation, and a slight decrease in appetite. The most pronounced changes were observed on days 10-15 from the beginning of the modeling of the disease in animals (day 26) in groups 1 (control) and 4 (diet with iodized salt).



**Figure 5.** Thyroid hormone levels in the blood serum of animals after simulation of hypothyroidism (day 50)

The levels of thyroid hormones in the sera of control animals consuming a diet with meat products containing no added iodine (group 1) and in group 2 (diet with meat products containing iodocasein) and group 3 (diet with meat products containing BioIod) animals were measured and compared, and the following can be stressed. In control animals (group 1), a significant decrease in T4 (1.8-fold) and T3 (2.3-fold) occurred; the level of TSH, on the contrary, increased 1.4-fold (Figures 4 and 5). In group 2 and 3 animals, consuming diets with iodine-containing meat products based on iodine-containing milk protein (casein) and iodine-containing whey protein, respectively, the serum levels of hormones changed only slightly after hypothyroidism was induced. A slightly better effect of iodine deficiency correction was achieved when the meat products were enriched with iodine-containing whey protein (BioIod; group 3) compared to the intact group, since the level of T4 was restored by 98.1%, T3 by 100% and TSH by 89.3% (Figures 4 and 5).

#### 4. Conclusion

In animals that consumed meat products enriched with iodized salt for 25 days, after the development of iodine deficiency, there was a decrease, due to iodine deficiency, in the serum thyroid hormones T4, T3 and TSH compared to intact animals. Enrichment of meat products with additives based on iodine-containing whey proteins and iodine-containing milk proteins (casein) has a protective effect in rats that are a model of iodine deficiency. It is likely that organically bound iodine is absorbed relatively well, thereby contributing to the development of an optimal amount of TSH and the formation of T4 in suitable amounts, compensating for the lack of iodine.

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# Fat replacement and PUFA enrichment challenges in fermented sausage production

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**Abstract.** Pork backfat is traditionally used in the formulation of dry fermented sausages and contributes to the properties of the final product. In addition to its important technological function during ripening and drying processes, this fat significantly affects the appearance, texture, and formation of the characteristic flavour and aroma of dry fermented sausages, so its substitution in these products is a major challenge for the meat industry. In order to produce reduced-fat fermented sausages with improved fatty acid composition, 16% of pork backfat was replaced with inulin gelled emulsions of corn or rapeseed oil. The addition of emulsions led to a significant decrease in saturated fatty acids and increase in polyunsaturated fatty acids, n-6 and n-3 types ( $P < 0.05$ ). An improved n-6/n-3 ratio was observed only in inulin-rape seed modified sausages (5.87). No signs of lipid oxidation measured by thiobarbituric acid reactive substance contents were detected in the modified sausages. However, the significantly higher total acid number and peroxide value in modified sausages ( $P < 0.05$ ) after ripening and 1-month storage indicate the greater susceptibility of these sausages to oxidation and lipolysis compared to control sausages.

## 1. Introduction

Europe is the major producer and consumer of dry fermented sausages which have a long tradition in Mediterranean countries, and a great variety of these products have been granted protected designation of origin (PDO) and/or protected geographical indication (PGI) labels [1]. Naturally fermented sausages are of great importance to the meat industry. These sausages are manufactured through traditional technologies from fresh pork meat and lard mixed with other ingredients (sugars, NaCl, black pepper, sweet and hot chilli pepper powder) that provide them with unique organoleptic and sensory profiles [2].

Even though fermented meat products have been consumed for centuries in different parts of the world and are considered to be one of the most valuable foods in human nutrition, the raw materials, meat and fat, used in the formulation of these products contain a higher proportion of saturated (SFA) than polyunsaturated fatty acids (PUFA) [3]. Numerous strategies have been developed in order to obtain new formulations of meat products with reduced SFA and cholesterol levels that provide different possibilities for the production of healthier fermented sausages [4]. When it comes to the enrichment of meat products with n-3 fatty acids, many studies were dedicated to a strategy that involved the increase in the proportion of these fatty acids (mostly  $\alpha$ -linoleic acid) in animal feed and



the consequent production of meat and fats rich in PUFA [5,6]. Other studies were based on an increased PUFA fraction in meat products by replacing pork backfat with soybean, fish or linseed oil, in order to increase the PUFA/SFA and decrease n-6/n-3 ratios [3,7,8]. The first vegetable oil used for these purposes in Greek fermented sausages, Chorizo de Pamplona (traditional Spanish fermented sausages) and soujouk (popular Turkish dry fermented sausages), was olive oil rich in monounsaturated oleic acid (56-87% monounsaturated fatty acid (MUFA)) [9,10,11]. In addition to olive oil, many vegetable oils abundant in 16:0, 18:1n-9 and 18:2n-6 such as soybean, linseed, grapeseed and corn oils were used in fermented sausage reformulation [5,7,12,13,14]. However, there are no data on similar use of rapeseed oil in fermented meat products. Rapeseed oil has a good potential as a pork backfat substitute, since it has moderate levels of 18:2n-6 and 18:3n-3, is rich in 18:1n-9 and has a 18:2n-6/18:3n-3 ratio of 2:1, which is considered beneficial for human health [15].

In most of the previous studies, different water/emulsion systems were used for incorporation of oils into the meat matrix. However, in order to preserve the texture of the product, there was a constant need for stabilization and structural strengthening of these emulsions, so the use of gelling agents (konjac glucomannan, alginates, agar, j-carrageenan, inulin, gelatine) helped to overcome the problems of reducing the hardness and water holding capacity of various reduced and low-fat meat products [13,14,16,17,18]. In this sense, recently our group [19] developed a gelled emulsion containing 20% linseed oil, 20% inulin and 2% gelatine as a pork backfat replacer in dry fermented sausages.

The objective of this study was to investigate the effects of pork backfat substitution with inulin corn or rapeseed oil gelled emulsions on the fatty acid composition and lipid oxidation parameters of dry fermented sausages after the ripening process and 1-month storage period.

## 2. Materials and Methods

### 2.1. Preparation of dry fermented sausages

Conventional dry fermented sausages (C) were prepared with 35% lean beef, 40% pork meat and 25% pork backfat, while in reduced-fat fermented sausages, 16% of pork backfat was replaced with inulin corn oil gelled emulsion (IC) or inulin rapeseed oil gelled emulsion (IR). To all three formulations, 23 g salt, 0.32 g curing salt, 4 g spice mixture (Cajna nova, Raps GmbH, Austria) and 0.25 g preparation of the ripening culture (FLORA ITALIA LC SafePro®, Chr. Hansen, Denmark) were added.

Inulin corn and rapeseed oil gelled emulsions were prepared in two phases according to Glisic *et al.* [19] with 150 g/kg water, 200 g/kg commercial corn (Uvita d.o.o., Serbia) or rapeseed oil (Suncokret d.o.o., Serbia) and 30 g/kg soybean lecithin for oil pre-emulsion, and 350 g/kg water, 20 g/kg pork gelatine and 250 g/kg inulin powder for inulin gelled suspension.

Meat, pork backfat and frozen inulin gelled emulsions were comminuted and mixed with all other ingredients in the cutter, after which the mixtures were stuffed into 50 mm diameter collagen casings and sausages were submitted to fermentation, cold smoking and ripening as previously described by Glisic *et al.* [19]. After ripening, sausages were aerobically packaged and stored at 4°C.

### 2.2. Chemical analysis

The fatty acid composition was determined in the lipid extracts of the sausages by gas chromatography. Hexane/isopropanol mixture (ASE 200; Dionex, Dreieich, Germany) was used for the accelerated solvent extraction of total lipids [20]. After evaporation of solvent until dryness under a stream of nitrogen, trimethylsulfonium hydroxide was used for fatty acid methyl ester (FAMES) preparation [21]. FAMES were separated and quantified by a gas chromatograph Shimadzu 2010 (Kyoto, Japan) with a cyanopropyl HP-88 capillary column (100 m × 0.25 mm × 0.20 µm) and flame ionisation detector. The identification of FAMES was done by comparison of the relative retention times of the peaks in the samples with those of standard pure compounds (Supelco 37 Component FAME Mix, Supelco, Bellefonte, PA, USA).

Acid values and peroxide values were respectively determined using standard methods ISO 660 [22] and ISO 3960 [23]. Thiobarbituric acid reactive substances (TBARs) were determined according to the combined method of Tarladgis *et al.* [24] and Holland [25], and are expressed as mg malonaldehyde/kg sample.

### 2.3. Statistical analysis

The entire trial was performed in triplicate. Six randomised sausages from each batch were analysed after the ripening period (day 28) and 1-month storage (day 58). Statistical analyses of the results were conducted using the software GraphPad Prism version 6.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The results were expressed as mean standard deviation. The differences between sausage were appraised by one-factor analysis of variance (ANOVA) with Tukey's multiple comparison test, while Student's *t*-test was performed to compare the content of docosanoic (C22:0), docosapentaenoic (C22:5n-3) and tetracosanoic fatty acid (C24:0) in two formulations after ripening. Differences considered significant if  $P < 0.05$ .

## 3. Results and Discussion

### 3.1. Fatty acid composition

Substitution of pork backfat with 16% inulin gelled emulsion of corn or rapeseed oil affected the fatty acid profile of modified sausages (Table 1). The content of myristic (C14:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), arachidic (C20:0), eicosenoic (C20:1) and eicosadienoic (C20:2) fatty acids was higher in IC and IR sausages ( $P < 0.05$ ). The most abundant fatty acid in IR sausages was oleic acid (C18:1cis-9). IR sausages also differed from C and IC sausages in the amount of  $\alpha$ -linoleic acid (C18:3n-3) ( $P < 0.05$ ). Similar changes in the fatty acid profile, especially in regard to the  $\alpha$ -linoleic acid content, were observed in sausage with inulin gelled linseed emulsion [19]. In IC sausages, the addition of emulsion significantly increased the content of linoleic acid (C18:2n-6) (20.44 g/100 g) as a consequence of its high proportion in corn oil [26]. These sausages also had the highest content of n-6 dihomog- $\gamma$ -linoleic acid (C20:3n-6). Docosanoic (C22:0) and tetracosanoic acids (C24:0) were detected only in reformulated products with emulsions. The contents of SFA were lower, while the PUFA and n-6 were higher in IC and IR sausages, and all parameters differed significantly in relation to C. The n-3 content significantly increased only in IR sausages (2.56), with the lowest n-6/n-3 ratio in these sausages (5.87) and the highest in IC sausages (19.57). The results from the present study are supported by other authors who also successfully improved the fatty acid profile and the nutritive value of modified fermented sausages by adding different vegetable oils in liquid, encapsulated, emulsified or gelled forms [5,7,8,12,13,14,16,18]. There are only a few studies concerning rapeseed oil emulsions as fat substitutes in meat batter for cooked sausages [27,28,29], while there are no data on the use of such emulsions in dry fermented sausage production. These results show the improvement in fatty acid composition of our fermented sausages with rapeseed oil addition, while IC sausages, apart from the observed differences in PUFA, as a result of an increase in n-6 content did not have a favourable n-6/n-3 ratio, with an increase of about 1.72-fold compared to control sausages.

**Table 1.** The fatty acid composition of control and fermented sausages with two different inulin gelled emulsions ripened for 28 days expressed in grams of fatty acid per 100 g of product

Fatty acid	Control sausages	Sausages with corn oil emulsion	Sausages with rapeseed oil emulsion
C14:0	1.43±0.03 <sup>a</sup>	0.91±0.04 <sup>b</sup>	0.80±0.07 <sup>c</sup>
C15:0	0.07±0.005 <sup>a</sup>	0.09±0.009 <sup>b</sup>	0.08±0.005 <sup>b</sup>
C16:0	24.69±0.42 <sup>a</sup>	19.12±0.48 <sup>b</sup>	18.58±0.37 <sup>b</sup>

C16:1	2.39±0.07 <sup>a</sup>	2.14±0.08 <sup>b</sup>	2.00±0.06 <sup>c</sup>
C17:0	0.36±0.005 <sup>a</sup>	0.38±0.03 <sup>a</sup>	0.38±0.016 <sup>a</sup>
C18:0	10.01±0.83 <sup>a</sup>	7.60±0.16 <sup>b</sup>	7.68±0.24 <sup>b</sup>
C18:1cis-9	44.37±0.81 <sup>a</sup>	44.65±0.50 <sup>a</sup>	50.25±0.20 <sup>b</sup>
C18:1cis-11	2.88±0.06	nd	nd
C18:2n-6	11.03±0.25 <sup>a</sup>	20.44±0.81 <sup>b</sup>	14.87±0.32 <sup>c</sup>
C20:0	0.22±0.01 <sup>a</sup>	0.29±0.027 <sup>b</sup>	0.32±0.009 <sup>c</sup>
C18:3n-3	0.46±0.04 <sup>a</sup>	0.57±0.05 <sup>a</sup>	1.75±0.09 <sup>b</sup>
C18:3n-6	0.03±0.005 <sup>a</sup>	0.03±0.005 <sup>a</sup>	0.03±0.005 <sup>a</sup>
C20:1	0.84±0.04 <sup>a</sup>	1.30±0.10 <sup>b</sup>	1.33±0.02 <sup>b</sup>
C20:2	0.36±0.07 <sup>a</sup>	0.57±0.01 <sup>b</sup>	0.55±0.02 <sup>b</sup>
C22:0	nd	0.25±0.02 <sup>a</sup>	0.21±0.03 <sup>b</sup>
C22:1+C22:4	0.27±0.05 <sup>a</sup>	0.23±0.02 <sup>a</sup>	0.26±0.05 <sup>a</sup>
C20:3n-6	0.08±0.01 <sup>a</sup>	0.88±0.09 <sup>b</sup>	0.09±0.01 <sup>a</sup>
C20:3n-3	0.10±0.03 <sup>a</sup>	0.09±0.03 <sup>a</sup>	0.13±0.02 <sup>b</sup>
C20:5n-3	0.38±0.018 <sup>a</sup>	0.06±0.005 <sup>b</sup>	0.16±0.031 <sup>c</sup>
C22:5n-3	nd	0.10±0.03 <sup>a</sup>	0.20±0.40 <sup>b</sup>
C22:6n-3	0.04±0.01 <sup>a</sup>	0.29±0.03 <sup>b</sup>	0.31±0.06 <sup>b</sup>
C24:0	nd	0.06±0.005 <sup>a</sup>	0.04±0.009 <sup>b</sup>
SFA	36.78±1.23 <sup>a</sup>	28.68±0.45 <sup>b</sup>	28.09±0.38 <sup>b</sup>
MUFA	50.48±0.96 <sup>a</sup>	48.09±0.58 <sup>b</sup>	53.58±0.14 <sup>c</sup>
PUFA	12.74±0.26 <sup>a</sup>	23.25±0.99 <sup>b</sup>	18.35±0.43 <sup>c</sup>
n-6	11.13±0.26 <sup>a</sup>	21.35±0.85 <sup>b</sup>	14.98±0.32 <sup>c</sup>
n-3	0.98±0.06 <sup>a</sup>	1.10±0.13 <sup>a</sup>	2.56±0.13 <sup>b</sup>
n-6/n-3	11.36±0.48 <sup>a</sup>	19.57±1.70 <sup>b</sup>	5.87±0.26 <sup>c</sup>

Means±SD with different lower-case superscript letters in the same row indicates differences (P<0.05); nd - not detected

### 3.2. Lipolysis and lipid oxidation

The lipid oxidation parameters of control sausages and sausages with corn and rapeseed oil inulin gelled emulsions are shown in Table 2. It is noticeable that reformulation led to differences in the degree of lipolysis after ripening. Specifically, the total acid number was significantly higher in reformulated sausages compared to C sausages (P<0.05), where IC sausages had the highest value (1.28 mg KOH/g) that differed from that of IR sausages (P<0.05). During storage, this value increased in all sausages, and the differences between modified sausages and control sausages remained (P<0.05), while the modified sausages did not differ among themselves for this parameter. The results obtained show the formulations with inulin gelled emulsions of oils rich in PUFA were more susceptible to lipid oxidation due to the generation of larger amounts of free fatty acids [30]. In our previous study, we also found a higher total acid number in sausages with inulin gelled emulsion of linseed oil [19]. Unlike our current results, Muguerza *et al.* [7] did not find any difference in the acid number of fermented sausages with increasing amounts of soybean oil added. Apart from higher PUFA content in modified sausages, these variations could be explained by different factors that affect the process of fatty acid formation by tissue and microbial lipases in dry fermented sausages, such as salt content, temperature and decrease in the number of microorganisms, or microbial lipolytic enzyme activity due to the decrease of  $a_w$  values [31].

**Table 2.** Lipid oxidation parameters of three different fermented sausage formulations after ripening (d 28) and after 1-month storage (d 58)

Days	Sausages	The total acid number (mg KOH/g)	Peroxide value (mmol/kg)	TBARs (mg malonaldehyde/kg)
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28	C	0.74±0.06 <sup>a</sup>	0.06±0.02 <sup>a</sup>	0.10±0.01 <sup>a</sup>
	IC	1.28±0.09 <sup>b</sup>	0.21±0.01 <sup>b</sup>	0.03±0.01 <sup>b</sup>
	IR	1.15±0.05 <sup>c</sup>	0.31±0.08 <sup>c</sup>	0.04±0.01 <sup>b</sup>
58	C	0.91±0.01 <sup>A</sup>	0.32±0.07 <sup>A</sup>	0.87±0.14 <sup>A</sup>
	IC	1.48±0.07 <sup>B</sup>	0.95±0.05 <sup>B</sup>	0.05±0.01 <sup>B</sup>
	IR	1.44±0.07 <sup>B</sup>	0.87±0.22 <sup>B</sup>	0.07±0.02 <sup>B</sup>

Means±SD with different lower-case superscript letters in the same column indicates differences ( $P<0.05$ ) after ripening (d 28); Means±SD with different upper-case superscript letters in the same column indicates differences ( $P<0.05$ ) after storage (d 58); C; control sausages; IC; sausages with inulin corn oil gelled emulsion; IR; sausages with inulin rapeseed oil gelled emulsion; TBARs; thiobarbituric acid reactive substances

Since lipolysis induces the oxidation and production of peroxides, similar changes were observed in the peroxide values of modified sausages. The peroxide number was significantly higher in IC and IR sausage formulations than control for both periods, after ripening (day 28) and storage (day 58) ( $P<0.05$ ). Previously published data showed also a higher peroxide number in fermented sausages with linseed oil gelled emulsion (0.29 mmol/kg) [19]. In contrast, in the study Muguerza *et al.* [7], the peroxide number was below the level of detection in sausages with added soybean oil and vitamin E. However, Alexandre *et al.* [14], in fermented sausages with konjac gel linseed oil emulsion as a fat substitute, recorded a peroxide number in the range of 0.33 to 0.38 mmol/kg, which is higher than in control and modified sausages from the present study after the ripening. These lower values of the peroxide number as well as the additional lipid stability of vegetable fats in our sausages with emulsions could be explained by the presence of soy lecithin for which it has been shown to exhibit good antioxidant properties [32].

It is interesting that during ripening and storage, reformulation did not lead to an increase in TBARs. IC and IR formulations had even significantly lower TBARs values than control ( $P<0.05$ ). These results are in accordance with other authors who determined TBARs below 1 mg malonaldehyde/kg in oil-modified fermented sausages after ripening and storage [5,8,14]. It can be considered that the stability of lipid oxidation and TBARs levels, especially during storage, in these fermented sausages is ensured by the addition of nitrite (nitrite content was significantly higher in IC and IR sausages compared to control – data not shown), using soy lecithin to prepare oil pre-emulsion and temperature of the refrigerator. Refrigeration temperatures reduce the rate of chemical reactions [33], nitrites react with C=C bonds of unsaturated fatty acids [34], and soy lecithin has an antioxidant effect in dry fermented sausages [32]. Lipid oxidation of unsaturated fatty acids is one of the main factors affecting the quality of meat and meat products, since it can lead to the development of off-odour and rancid taste due to the formation of compounds such as n-alkenals and dienals, drip losses, discolouration, nutrient loss, reduction of shelf-life and accumulation of mutagens and carcinogenic compounds [35]. It is confirmed that lipid oxidation products are associated with the aetiology of various neurodegenerative and cardiovascular diseases, as well as some types of cancers [13]. Considering this, it is important not only to improve the nutritional value of meat products but also to minimize the degree of lipid oxidation.

#### 4. Conclusion

Taking into account established eating habits and consumers' preferences for recognizable meat products, the results of this study show it is possible to formulate such product with reduced SFA, increased PUFA content and a low n-6/n-3 ratio. Reformulated sausages could be a good alternative to classic foods that are sources of dietary long-chain PUFAs. Furthermore, the inulin gelatine network and soy lecithin have the potential to be a good oil-stabilizing matrix that could reduce lipid oxidation in PUFA-enriched dry fermented sausages. However, since this oil carrier was not that effective in sausages with linseed oil gelled emulsion, further research is needed to confirm these findings.

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## Factors influencing mussel (*Mytilus galloprovincialis*) nutritional quality

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**Abstract.** Mussels display interesting nutritional characteristics as they are a rich source of proteins, carbohydrates and minerals and provide an almost unlimited variety of fatty acids with beneficial roles in human health. The quality characteristics of *Mytilus galloprovincialis* mussels harvested at seasonal intervals reflect the different environmental conditions met by the animals during their growth. Their chemical composition is strictly dependent on the phytoplankton resources available and, therefore, on the season of harvest. Parameters such as water temperature, food availability and the gametogenesis cycle can influence the meat yields and the biochemical composition of the mussels, conditioning their commercial quality and organoleptic characteristics. In order to determine the nutritional value of blue mussels, it is of great relevance to identify their biochemical composition as well as the most favourable season and geographical location for mussel-harvesting. That data could be useful to indicate the periods of the year more suitable for the marketing and consumption of mussels.

### 1. Introduction

In many countries, mussels (*Mytilus galloprovincialis*) are considered a delicacy and important marine organisms due to their nutritional relevance. They are an important part of the global seafood market and support both commercial fisheries and aquaculture all around the world [1]. Mollusc aquaculture accounts for more than 75% (13.9 million tons) of the world's aquaculture, with mussel production being around 13% (1.8 million tons) of world aquaculture annual production [2]. Mussels are usually marketed as raw or frozen but also as processed products, i.e. smoked mussels. Consumption of fresh mussels is growing owing to their high nutritional quality. Mediterranean mussel farmers are increasingly interested in technologies to obtain high quality final products, so high quality of raw material is essential. Mussels are appreciated by consumers for their organoleptic properties, retained also after processing, and for their competitive price if compared with other bivalves [3].

#### 1.1. Biochemical composition of mussels

Mussels are filter-feeders, acquiring proteins, lipids, carbohydrates and other components from phytoplankton (e.g., diatoms, dinoflagellates), bacteria and detritus suspended in the water, and using them to build their own biomass [4]. The main factors of molluscs' nutritional value are proteins, lipids, carbohydrates, free amino acids, vitamins (A, B1, B2, B6, B12 and C), fatty acids, particularly



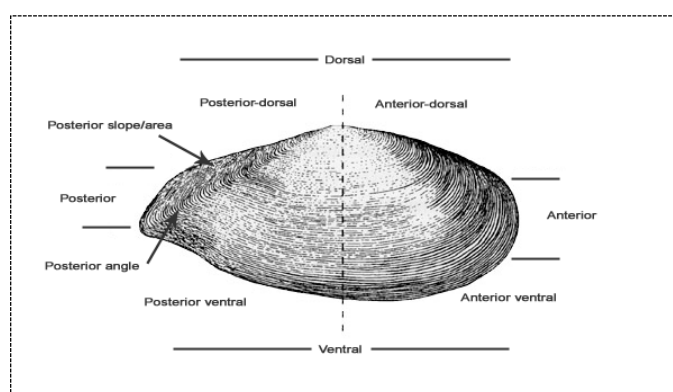
the n3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic (EPA, 20:5n3) and docosahexaenoic (DHA, 22:6n3), which clearly shows their importance for human health [5]. They play an important role in prevention of cardiovascular disease, regulation of blood pressure and the immune system and have anti-inflammatory properties [6].

The biochemical composition, condition index and meat yield are useful indicators of the nutritional and commercial quality of bivalves. These parameters are affected by season and capture areas and fluctuate as a result of the interaction between the variations in the seston (the natural diet of suspension-feeders) quality and quantity and the bivalves' reproductive cycle [7,8]. The reproductive cycle of *M. galloprovincialis* starts with the development and ripening of the gonad during autumn-winter, and major spawning events take place during the spring upwelling season, after which the gonad restores, leading to secondary spawning events during late summer. The most important ecological factor affecting the rate of growth is food (different groups of phytoplankton), the composition of which can be different in differing aquatic environments. Other important factors are temperature and salinity of seawater. The largest amount of food in the sea and the greatest mussel growth is achieved at temperatures from 10 to 20°C. At temperatures above 20°C and below 5°C, the growth increment is usually slow [9].

In addition to seasonal variations, spatial changes in the feeding environment are common in harvesting areas. Storms or tidal cycles, changes in current speed or variable riverine nutrient outflow can lead to resuspension of bottom material, which increases the total concentration of seston, but dilutes the concentration of organic particles suspended in the water [10]. Human activities can reduce organic loads and chlorophyll levels [10]. Numerous studies have reported the amount of biochemical reserves and the condition index can vary substantially among bivalves cultured in nearby sites within the same embayment [8,11,12].

### 1.2. Biometric parameters

Differences in biometric parameters have a direct influence on the aspect of mussels and can be decisive for consumers' purchase decisions. Biometric parameters are measured in individual mussels using 0.05 mm precision callipers: length (maximum measure along the anterior-posterior axis), width (maximum lateral axis), and height (maximum dorso-ventral axis) (Fig. 1). Mussels are opened by cutting the adductor muscle with a scalpel, and the wet meat and shells are weighed.



**Figure 1.** Biometric parameters of mussels: length (maximum measure along the anterior-posterior axis), width (maximum lateral axis), and height (maximum dorso-ventral axis).

The biometric measurements of mussels throughout all seasons did not exhibit seasonal differences in terms of total weight, length, height or width. Mussel shell contains little organic material (<5%) and changes in biometric characteristics of the shell are less susceptible to food availability and other environmental factors than mussel tissue [13].

Meat yield (MY), i.e. the percentage ratio between meat content (WT) and total wet weight of mussels (WW) is an important aspect of marketability of mussels and is calculated according to Okumus and Stirling [14] as follows:

$$MY (\%) = (\text{meat weight (g)} / \text{whole mussel weight (g)}) \times 100$$

Condition index (CI) is calculated after oven-drying (105°C) meat and shell according to [7] as follows:

$$CI (\%) = (\text{meat dry weight (g)} / \text{shell dry weight (g)}) \times 100.$$

In Wales, the largest gains in flesh weight occur between April and September, which corresponds to the season of maximum shell growth [15]. The nutritional quality of the seston peaks during the spring bloom and decreases during winter downwelling. Variations in CI are significantly correlated with the accumulation and expenditure of reserves. Mussels harvested in autumn have the highest CI and biochemical reserves, while minimum CI occurs in winter, when mussels have a low energy balance, high energy investment during the typical winter gonad development and low food quality. Thus, some results suggested that mussels harvested in spring and summer (spawning period) would be more suitable for processing, while the higher CI during autumn indicates the mussels harvested during this period are more suitable for fresh consumption [16].

### 1.3. Chemical analyses

For proximate chemical composition studies, chemical analyses are performed on a homogenized sample of about 10 individual mussels. Pooled mussels can be freeze-dried, milled and kept in dry conditions until further analysed [17]. The moisture and ash contents are determined according to the standard procedures of AOAC [17]. Moisture content is calculated based on the percentage weight loss after drying to a constant weight at 105°C overnight. Ash content is determined after having weighed and transferred the dry samples into a muffle furnace at 550°C overnight. Quantitative protein determination is performed by the Kjeldahl method ( $N \times 6.25$ ) from the nitrogen concentration of mussel. Lipid content is determined gravimetrically after Soxhlet extraction using petroleum ether. The lipid extracts are subject to fatty acid analysis by gas chromatography after transmethylation into fatty acid methyl esters (FAMES). Glycogen content ( $\text{mg g}^{-1}$  of wet weight) can be measured by colorimetric reaction and calculated based on a mussel glycogen standard (Sigma, Saint Louis, MO) following the methodology reported by Gallardi *et al.* [18].

### 1.4. Seasonal variation

During the year there is a change in the biochemical composition of shellfish. Stress conditions, environmental situations requiring major energy expenditure and gamete release can be responsible for the low condition index, low meat yield and poor biochemical reserves observed in certain periods of the year [15]. Proteins have many different biological functions: transport, defence, structural elements, storage. They are considered the main energy substrate during gamete development, and the lowest protein levels in mussels are coincident with the main periods of gamete development (winter) and spawning (spring), respectively. Decreases in lipid content in mussels have also been associated with gamete formation and spawning effort (winter). Lipids are highest during spring-summer, when peak values coincide with the phytoplankton bloom [19]. In human diets, lipids have several important roles: provision of a significant proportion of the body's necessary energy requirements, as cell membrane components, and being the source of essential fatty acids [20].

Glycogen makes up more than 50% of the total carbohydrate reserves in bivalves and has two major functions: as a long-term energy store and as structural elements with lipids. An accumulation

of glycogen over the summer season, followed by a decline through the winter is coincident with low meat yields and low condition index and with spawning, was reported by Okumus and Stirling [14] in mussels. The low meat yield and low condition index in winter are coincident with the depletion of protein and lipid reserves. The ash content of the meat reaches peak levels in winter, coincident with minimum glycogen and lipid reserves.

Gas chromatographic analysis of total lipids in mussels shows the prevalence of polyunsaturated fatty acids over saturated and monounsaturated fatty acids occurs throughout the year [7]. The fatty acid composition (among the total fatty acid content) of mussels varies, ranging from 29% to 48% of PUFA, 16 to 32% of monounsaturated fatty acids and 23 to 45% of saturated fatty acids, although saturated fatty acids accounted for 57% of the fatty acids in mussels in Italy [21].

In summary, seasonal variations in the biochemical composition of mussels are closely linked to natural fluctuations in the composition of the bivalve's diet and the different stages of the annual reproductive cycle. These facts could benefit aquaculturists and local residents employed in entrepreneurship related to aquaculture, and are relevant in developing a bivalve trade that ensures a constant supply of high quality product.

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# Effect of the direction of *m. psoas major* fibres on the results of tensile test - can we model meat as a material?

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**Abstract.** The aim of this study was to examine the possibility of tensile-test application at three strain rates (0.01/s and 0.001/s and 0.001/s) on suitable samples of grilled pork meat (*musculus psoas major*). Differences in the stress-strain curves were observed between the two directions of the muscle fibres (i.e. strain parallel to and transverse to the fibres). However, the strain rate of 0.001/s resulted in the most linear stress-strain curves for strain in both muscle fibre directions. Also, results confirmed that specimens tested transversally to the muscle fibre direction required less stress to fracture. We also concluded that specimens stretch more in the direction transverse to the muscle fibre direction for strain rates of 0.01/s and 0.001/s. Gaining knowledge from different methods of empirical mechanical testing of meat should enhance the possibility of forming material constitutive laws to be used as input to finite element simulations of industrial processes of meat such as cutting or of human oral processing.

## 1. Introduction

Oral processing from the moment of the first bite to the moment of swallowing includes mechanical changes of food structure during mastication, chemical changes related to oral enzymatic digestion, and temperature-associated transitions such as melting [1, 2]. Starting from the first bite to the moment of swallowing, food changes are caused by rhythmic motor activity of the jaw controlled by the central nervous system, and transportation of the food pile in the oral cavity by the tongue movements [1, 3]. The pathway of nonliquid food structure changes during mastication includes mechanical structure failure, further grinding of food particles, saliva incorporation, particle agglomeration, and bolus formation [4, 5].

The mechanical characteristics of food determine its behaviour as a material during oral processing, which means they determine such parameters as particle size distribution, eating rate, number of chews per gram, etc. Besides that, palatability of the food can affect food digestion, absorption of nutrients and the occurrence of disorders such as obesity or dyspepsia [4, 6]. Some authors have confirmed that denture wearers do not have the same patterns of meat mastication compared to fully dentate masticators [7].

Although the mechanical characteristics of food can be of great importance, there are not many studies that deal with the mechanical testing of meat. One of the reasons for this is that meat has a complex structure and so is considered as a composite and anisotropic material [8], which means that, for mechanical testing, it is hard to obtain appropriate samples consisting just of muscle tissue [9]. Also, we must bear in mind that other materials such as connective and adipose tissues are present in the meat



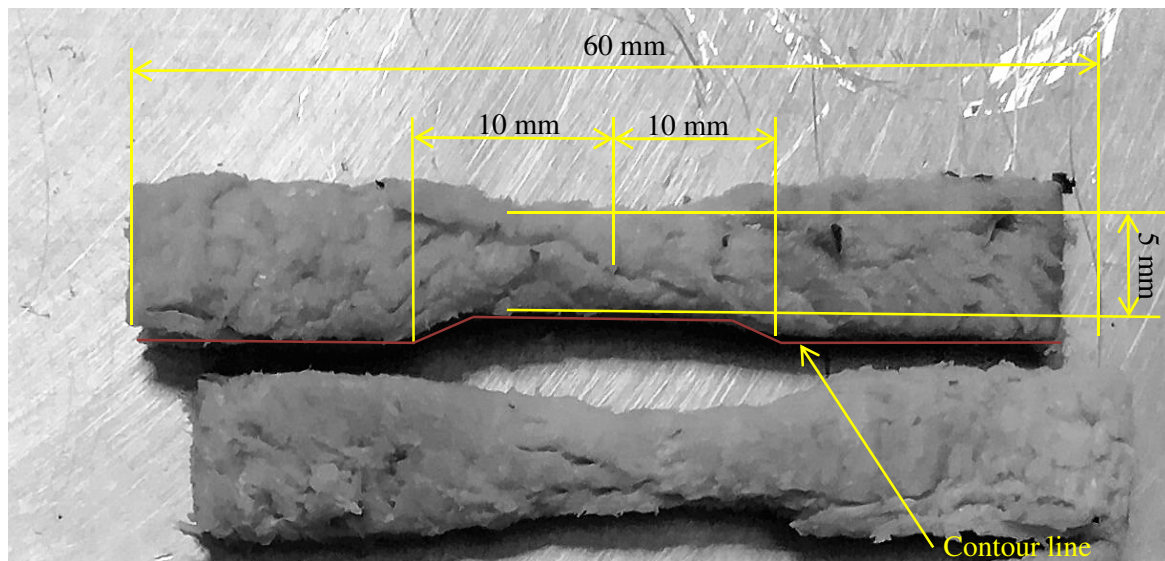
as well. An additional factor that can affect the results of mechanical testing is the direction of the muscle fibres [8, 9]. Besides that, other factors such as: breed, feeding regime, sex, age, animal treatment before slaughter and carcass management can have an influence on meat toughness [8].

In the case of meat, Honikel [10] suggested the tensile test, Warner-Bratzler shear test and penetrometer measurements as appropriate methods of meat tenderness evaluation. Lepetit and Culioli [9] pointed out the suitability of the following mechanical methods for meat toughness examinations: Warner-Bratzler test, compression test, tensile test, penetrometry, multi-blade shearing, and bite test.

One of the latest trends in food science and engineering is mechanical characterization and modelling of food materials [11, 12]. Data obtained from these types of mechanical tests are needed for the definition of the material constitutive laws and can be inputs for further modelling of the material. In this study, the possibilities for mechanical testing of grilled pork meat (*musculus psoas major*) were investigated in terms of tensile tests. The working hypothesis was that results differ depending on the direction of muscle fibres.

## 2. Materials and methods

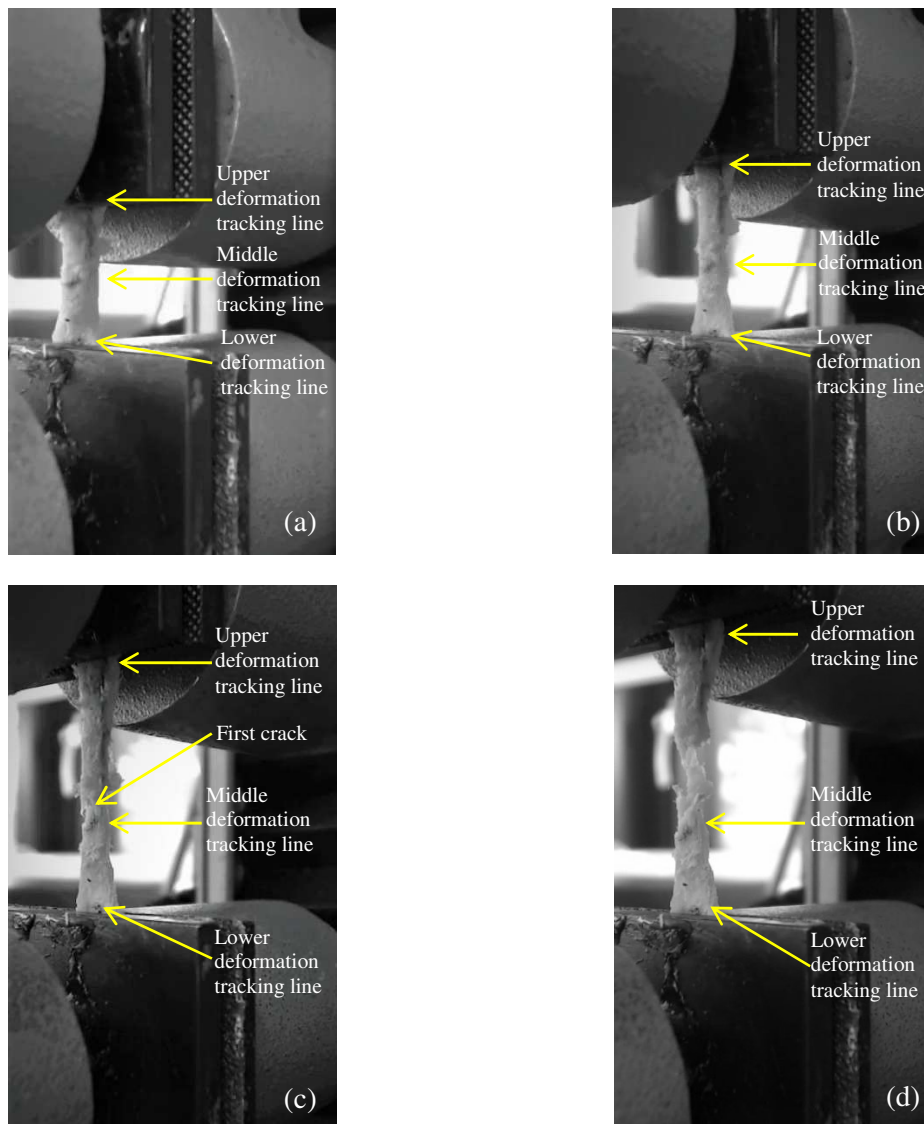
Pork meat used for this study (*musculus psoas major*) was commercially purchased at a London market. Preliminary tests were conducted on raw meat. Even though Honikel [10] considered it possible to conduct a tensile test on raw meat, our preliminary trials for this study showed it is hard to obtain uniform geometry and dimensions of tensile test specimens of raw meat. Therefore, in order to produce samples with adequate geometry, the meat was grilled. Figure 1 shows the dumbbell shape of the specimens for uniaxial tension, denoting dimensions. Dimensions were chosen according to recommendations provided in the literature [10].



**Figure 1.** Dumbbell-shaped specimens for uniaxial tension test, parallel to the muscle fibre direction

Meat (3 mm thick slices) was grilled then cut with a razor blade in order to gain specimens as shown in Figure 1. Specimens were cut from the grilled meat with a metal cutting template parallel to the direction of muscle fibres and transverse to the direction of muscle fibres. Hence, two types of specimens were obtained for the tensile tests.

Before tensile testing, the dimensions of all grilled meat specimens were measured with a digital Vernier caliper of 0.1 mm accuracy. Specimen measurements were as follows: height:  $19.00 \pm 1.50$ ; width:  $4.91 \pm 0.74$ ; thickness  $3.49 \pm 0.77$ . They were labelled with a marker at three points along the gauge length in order to track uniformity of specimen deformation during the test. For this purpose, all tests were video recorded (Figure 2).



**Figure 2.** Specimen deformation during the tensile test, parallel to the muscle fibre direction, 0.1/s constant strain rate; (a) specimen before the beginning of the test; (b) tension of the specimen; (c) first crack of the specimen; (d) specimen material failure

Tensile tests were conducted on an Instron machine 2530. All tests were performed with the 10 kN load cell. The strain rate dependency was assessed via monotonic tests at three constant true strain rates: 0.001/s, 0.01/s, and 0.1/s. For each of the two different muscle fibre directions and each of three strain rates, five replicates were conducted. All data are shown below as mean values, using standard deviation as error bars. The true (Cauchy) stress,  $\sigma$ , *versus* true (Hencky) strain,  $\varepsilon$ , were calculated using the following equations:

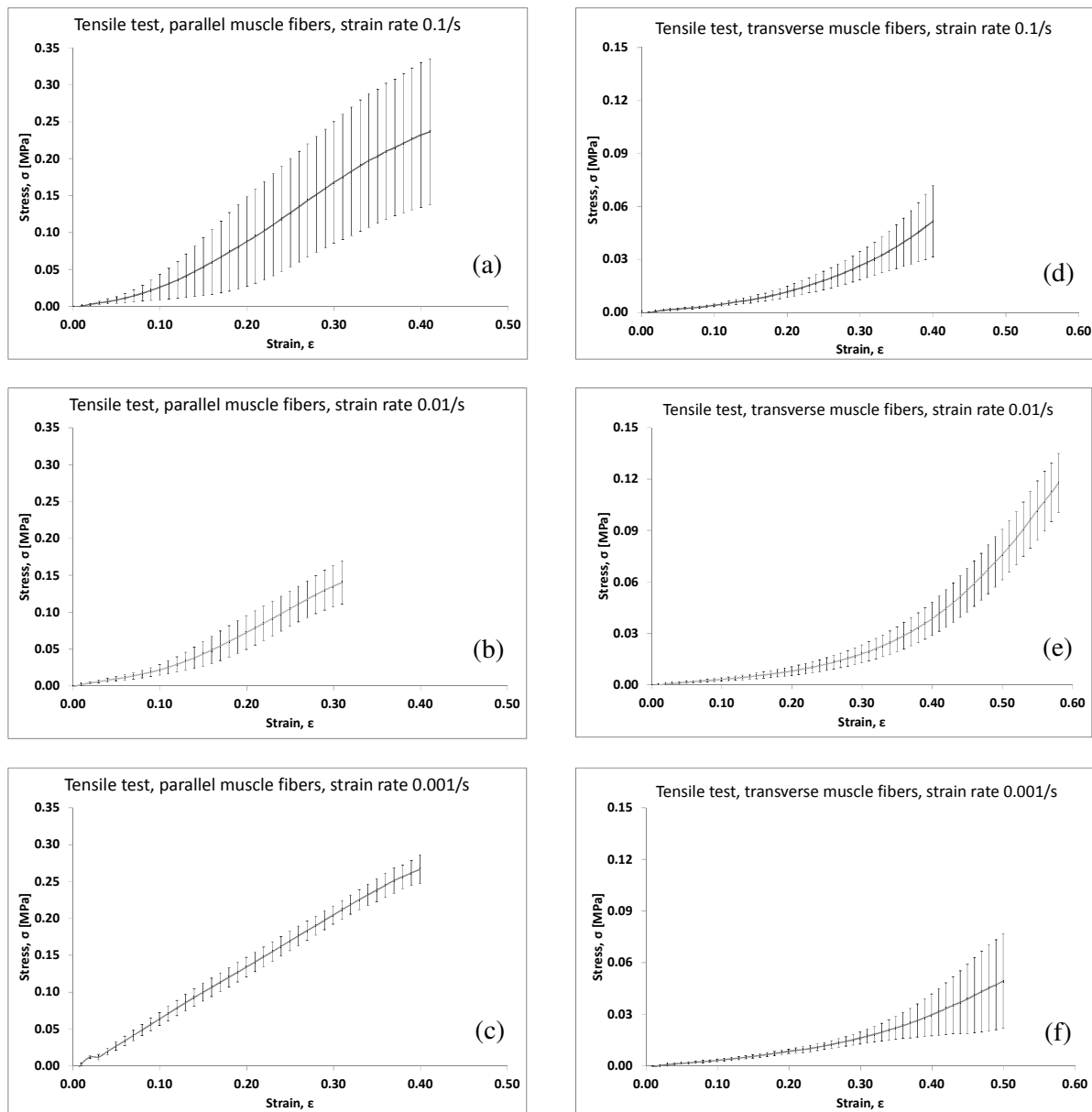
$$\begin{aligned} (a) \quad & \sigma = F/A_i \\ (b) \quad & \varepsilon = \ln(H_i/H_o) \end{aligned} \quad (1)$$

where  $A_i$  is the instantaneous cross-sectional area of the specimen,  $F$  is the corresponding force applied and  $H_o$  is an original reference dimension of the specimen (gauge length in tension) together with its deformed value,  $H_i$ .

Tests were performed in a laboratory maintained at 23°C and 50% relative humidity.

### 3. Results and discussion

Tensile tests resulted in six stress-strain diagrams, three for each of the muscle fibre directions. For both of the directions, three tensile tests were conducted at 0.1/s, 0.01/s, and 0.001/s constant strain rate (Figure 3). Even though video analysis of the testing was employed, with the aim of excluding unsatisfactory data from further data analysis, the diagrams show significant variability in the stress-strain data. Still, differences in the stress-strain curves can be noted between the two directions of the muscle fibres.



**Figure 3.** Stress-strain curves; (a) monotonic tension, 0.1/s strain rate, parallel to the direction of the muscle fibres; (b) monotonic tension, 0.01/s strain rate, parallel to the direction of the muscle fibres; (c) monotonic tension, 0.001/s strain rate, parallel to the direction of the muscle fibres; (d) monotonic tension, 0.1/s strain rate, transverse to the direction of the muscle fibres; (e) monotonic tension, 0.01/s

strain rate, transverse to the direction of the muscle fibres; (f) monotonic tension, 0.001/s strain rate, transverse to the direction of the muscle fibres

Comparing the diagrams, it appears the strain rate of 0.001/s resulted in the most linear stress-strain curves for both muscle fibre directions. From Figure 3, it can be concluded that specimens tested transversally to the muscle fibre direction required less stress to fracture. On the other hand, by considering Figures 3b, 3c, 3e and 3f, for the strain rates of 0.01/s and 0.001/s, it appears the specimens of grilled meat stretched more in the case of the transverse muscle fibre direction.

Grilling the meat improved the shape and uniformity of the specimens' dimensions, but further enhancements are still needed. Because of the material cracking between the muscle fibres when the specimens were cut from the grilled meat, future testing should reconsider the replacement of metal cutting templates with a suitable manual press. Also, the drying of the specimens during the tests should be considered.

### 3.1. *Future modelling of the results*

There are two basic approaches in modelling meat as a material. The first is reproducing food separation patterns under strain tests, which could resemble boundary conditions applied when modelling the chewing of meat using finite element analysis [11, 12]. This should allow information on the mechanical behaviour of meat to be obtained. Such information could enhance better our understanding of the structural changes of meat during oral processing.

The second approach is introducing mechanical characteristics of meat as oral processing parameters in order to model specific quality parameters, using quality function deployment [13], or quality index models [14]. This type of modelling can enable quality improvement aimed at satisfying the consumers as well as translating the consumer's oral processing demands into meat quality targets.

Recent research conducted by Dekkers et al. [15] deals with structuring of meat analogues. It can be assumed that better understanding of meat's mechanical properties would improve the structure of meat analogues. Some recent studies are introducing novel technologies in the area of food science and engineering, such as 3D food printing. Most of these studies are investigating the applicability for 3D printing of plant origin materials such as potato starch [16], pectin [17] or xanthan gum [18]. Gaining knowledge of meat mechanics and related structure characteristics should lead to future progress in the design of foods through 3D food printing, which is a promising area in the field of food engineering.

## 4. Conclusion

Literature findings point to the possible difficulties regarding mechanical testing of meat. Although some authors maintain it should be possible to conduct mechanical tests on raw meat, preliminary studies for the present research revealed this type of testing as inappropriate. On the other hand, limited scientific findings analysed mechanical testing of grilled meat.

With the aim of examining the uniaxial tension of grilled pork meat, monotonic tensile tests with constant strain rates were performed for two different muscle fibre directions and three different strain rates. This research revealed differences in the stress-strain curves between the two directions of the muscle fibres. The strain rate of 0.001/s resulted in the most linear stress-strain curves for both muscle fibre directions examined. Also, this study showed that the strain testing transversally to the muscle fibre direction required less stress for the muscle fibres to fracture.

This study highlights the need for understanding various breakdown mechanisms during chewing of meat as one of the most complex food materials. Modelling should try to utilize food disintegration and bolus breakdown and its relation with the original mechanical and physical properties of meat. Further research could cover various types of meat and chemical changes that occur due to saliva incorporation or enzymatic gastric conditions. Meat structure and related mechanical characteristics are required as inputs to novel food engineering methods such as 3D printing of food.

## Acknowledgement

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# The influence of cooking methods and juniper essential oil on lipid oxidation in pork chops

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**Abstract.** In this research, the effects of three cooking methods (boiling–vacuum bag, boiling–plastic bag and baking) on the lipid oxidation and overall sensory acceptability of pork chops marinated with and without juniper essential oil were investigated. The thiobarbituric acid reactive substance content was affected by both marinating and cooking methods. Pork chops from juniper essential oil marinade had lower thiobarbituric acid reactive substance values throughout the marinating and two cooking methods (boiling–plastic bag and baking) in comparison to their control counterparts. The pork chops with juniper oil, regardless of cooking method, were evaluated with significantly higher scores for overall acceptability than all analysed control pork chops.

## 1. Introduction

Different cooking methods are applied to meat to improve its hygienic quality by reducing the number of pathogenic microorganisms and extending shelf life [1]. This is accompanied by enhanced palatability, tenderness and flavour [2,3]. However, cooking causes physico-chemical changes in protein, carbohydrate, lipid and other minor components, thus altering the nutritional value of meat [1,4,5]. Lipid oxidation occurring during cooking is the main reason for quality deterioration of meat and meat products [6]. The rate and level of lipid oxidation could be affected by cooking procedures (temperature, time, moist or dry heat) [2,3,7].

In order to prevent oxidative rancidity and increase meat products' shelf-life, numerous natural products containing phenolic compounds have been used to replace chemical antioxidants which could have harmful effects on people's health [8,9,10,11]. Recent investigations showed that essential oils and extracts from various herbs and spices provide a good alternative to synthetic antioxidants [12, 13,14,15].

Juniper (*Juniperus communis* L.) is a plant belonging to the family Cupressaceae. It is defined as a small coniferous long-lived tree or an evergreen shrub and is distributed throughout Europe, North Asia, and North America. Common juniper has been used as medicinal plant for centuries. Juniper oil was applied in the treatment and prevention of cholera, helminthiasis and renal infections. Also, it has been used in gastronomy as a spice for meat and meat products and as a natural ingredient in cosmetic, pharmaceutical and food industries [16,17]. Nowadays, the cones of *J. communis* and their essential oils are recognized by the European Pharmacopoeia [18,19,20].





There is a scarcity of data regarding the effect of juniper essential oil on lipid oxidation in meat and meat products. Therefore, the aim of this study was to investigate the impact of juniper essential oil along with different cooking methods (boiling and baking) on lipid oxidation in pork meat.

## 2. Experimental part

### 2.1. Raw materials

Fresh whole pork loins (*M. longissimus thoracis et lumborum*) were obtained from a local supermarket. The chosen pork loins were of normal meat quality, regarding criteria for pH (pH <6.0) and lightness (CIE  $L^*$  value = 43-50). All external fat, fascia and separable connective tissue were removed. Thereafter, pork loins were cut perpendicular to the longitudinal orientation of the muscle fibres into 1 inch thick pork chops. The *Juniperus communis* essential oil used in this study was of commercial origin.

### 2.2. Marinating of pork chops

Two different marinades were prepared for the current study. Both marinades consisted of 2.2% salt in water solution and were in the proportion of 15 g marinade per 100 g meat. Juniper essential oil was used in marinade at a concentration of 20  $\mu$ l per 100 g meat (JEO). Water with salt and no essential oil was used as a control (C). Both marinades were applied by massaging during 20 minutes.

### 2.3. Cooking procedure

The applied cooking procedures were: two boiling procedures (boiling – each pork chop was placed in a vacuum sealed plastic bag or packed into a plastic bag and totally immersed in an electric water bath at 90°C until the final internal temperature of 72°C was achieved), and; baking (each pork chop was cooked on a grill in an electric air-convection oven preheated to 163°C until 72°C was reached in the centre of the sample). After the cooking, the pork chops were cooled at room temperature and used for further analyses.

### 2.4. Thiobarbituric acid–reactive substances (TBARS) determination

The extent of lipid oxidation was assessed by the TBARS method of Botsoglou *et al.* [21], with modifications described by Šojić *et al.* [22]. Results were expressed as mg malondialdehyde (MDA) per kg of meat.

### 2.5. Sensory analysis

Sensory analysis was carried out by eight trained panellists. Cooked pork chops were cut into 1.0 × 1.0 × 1.0 cm pieces and evaluated for overall acceptability on a nine-point scale (1 – dislike extremely, 2 – dislike very much, 3 – dislike moderately, 4 – dislike slightly, 5 – neither like nor dislike, 6 – liked slightly, 7 – liked moderately, 8 – liked very much and 9 – like extremely).

### 2.6. Statistical analysis

Statistical analysis was carried out using Statistica 13.0 (TIBCO Software Inc., Palo Alto, USA) one-way and two-way ANOVA. One-way ANOVA was used for analysis of the overall sensory acceptability of cooked pork chops. Two-way ANOVA was used for analysis of TBARS as a function of the marination (C and JEO) and of the cooking method (boiling-vacuum bag, boiling-plastic bag and baking). Duncan's test was used to identify significant differences among main effects (marination and cooking methods). Significances were established at the level of  $P < 0.05$ .

## 3. Results

Lipid oxidation of raw marinated C and JEO pork chops measured as TBARS was, on average, 0.07 and 0.03 mg MDA/kg, respectively. Raw JEO pork chops had significantly ( $P < 0.05$ ) lower TBARS values compared to C pork chops, which could be related to the presence of added juniper essential



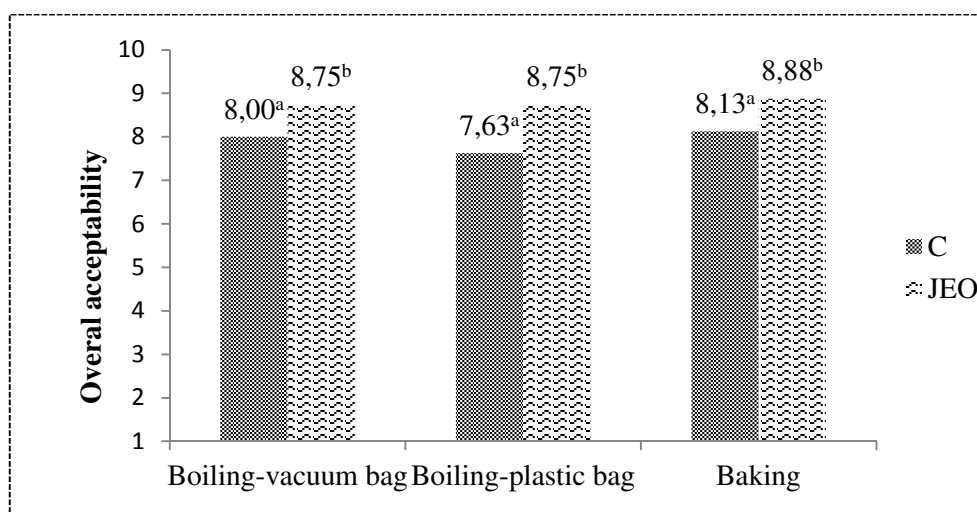
oil. The effect of different marinade formulations and cooking methods on lipid oxidation of the cooked pork chops is presented in Table 1. As was expected, all cooking methods significantly ( $P < 0.05$ ) increased TBARS values. Similar results were obtained by several other authors [2,23,24]. The increase in TBARS values after the applied cooking methods could be due to the high temperatures that promoted oxidation processes, thus increasing the levels of thiobarbituric reactive substances [1,4].

**Table 1.** Thiobarbituric reactive substance values (mg malondialdehyde/kg) of cooked pork chops as affected by cooking method and marinade formulation and significance of interactions between marinade type and cooking method

	Control pork chops	Pork chops with juniper essential oil	Two-way ANOVA		
			Marination effect P	Cooking method effect P	Marination $\times$ cooking method interaction P
TBARS (mg			0.000044	0.000001	0.003360
Boiling – vacuum bag	1.29 <sup>a1</sup>	1.14 <sup>b1</sup>			
Boiling – plastic bag	1.20 <sup>a2</sup>	0.94 <sup>a1</sup>			
Baking	1.59 <sup>b2</sup>	1.11 <sup>b1</sup>			

<sup>a,b</sup> Different letters indicate significant differences ( $P < 0.05$ ) between pork chops following the same marinade formulation<sup>1,2</sup> Different numbers indicate significant differences ( $P < 0.05$ ) between pork chops following the same cooking methods

Cooking causes membrane disruption that releases prooxidant substances, such as non-haem iron, thereby accelerating the oxidative processes [25]. Application of high temperature (163°C) during baking generated significantly ( $P < 0.05$ ) higher TBARS values than at 90°C (boiling) for C pork chops (1.59 and 1.29 or 1.20 mg MDA/kg, respectively). Similar results were found by other authors [1,2,4,24]. However, there was no significant ( $P < 0.05$ ) difference between JEO pork chops prepared by boiling-vacuum bag and baking, regarding TBARS values (1.14 and 1.11 mg MDA/kg, respectively). These values were significantly ( $P < 0.05$ ) higher than those of JEO pork chops cooked by boiling-plastic bag (0.94 mg MDA/kg). Significantly lower TBARS values were measured in JEO marinated pork chops prepared by boiling-plastic bag and baking (0.94 and 1.11 mg MDA/kg, respectively) compared to their counterparts from control groups (1.20 and 1.59 mg MDA/kg, respectively). This result could be explained due to addition of juniper essential oil that retarded lipid oxidation during cooking [26]. The major components in the composition of juniper essential oil are  $\alpha$ -pinene, sabinen,  $\beta$ -pinene, limonene and myrcene [17,20]. However, the antioxidant activity of juniper essential oil could be attributed to its components  $\alpha$ -terpinene and  $\gamma$ -terpinene that are present in lower concentrations [19].



**Figure 1.** Overall sensory acceptability of marinated cooked pork chops

The results of the sensory analysis revealed a significant ( $P < 0.05$ ) difference for overall sensory acceptability between pork chops treated with different marinades (Figure 1). The JEO pork chops cooked by baking were the most acceptable, but their scores were not significantly ( $P < 0.05$ ) different from JEO pork chops cooked by boiling–vacuum bag and boiling–plastic bag (8.88, 8.75. and 8.75, respectively). Also, there was no significant ( $P < 0.05$ ) difference among the sensory score values of C pork chops.

#### 4. Conclusion

Cooked pork chops are highly susceptible to lipid oxidation due to the applied heat treatment. Results in this study show that juniper essential oil can retard lipid oxidation in raw and cooked pork meat. Also, juniper essential oil has a positive effect on overall sensory acceptability of cooked pork chops. Usage of herb essential oils and extracts which possess antioxidant properties could retard lipid oxidation and prevent loss of nutritional value of food.

#### Acknowledgement

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# Sodium intake associated with meat product consumption in Serbia

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**Abstract.** Meat and meat products are an important part of the human diet in Serbia. They are important because of their desirable taste, high nutritional value, variety of the products, traditional considerations etc. Our study addresses sodium levels in some of the most commonly consumed meat products from the Serbian market (cooked sausages, dry fermented sausages, smoked meat products, canned meats and pates). Sodium content was determined using ICP-MS. The highest sodium level was established in dry fermented sausages, and the lowest content was measured in pate. No significant differences were seen in sodium levels between cooked sausages, smoked meat products and canned meats. The mean sodium level of all analysed meat products was 11.8 g/kg. According to average intake of meat products in Serbia (54.7 g/day) and the measured sodium levels, consuming these meat products could contribute up to 26.9% of sodium daily requirements. This could pose a risk to human health considering the already high sodium intake through adding salt to food and consumption of various processed foods. Generally, the results obtained suggest reducing salt levels in meat products and as well as in diet in general.

## 1. Introduction

Meat products are manufactured predominantly with sodium chloride as it provides salinity, flavor and increases shelf life. Therefore, these products are significant sources of sodium in human diets [1]. Sodium is also one of the essential nutrients that has an important role in maintaining some biochemical and physiological functions in the body [2]. However, excessive sodium intake has been correlated to risk of increased blood pressure and it could directly lead to heart attack [3-5]. Beside cardiovascular diseases, increased sodium intake has been linked with other health problems, including sodium retention in extracellular fluid [6], bone density disorders [7], risk of gastric cancer [8], proteinuria and a risk of kidney calculus [9].

Considering the relationship between sodium intake and various chronic diseases, the National Institute of Health [10] established dietary reference intakes (DRIs) for sodium: recommended dietary allowances and adequate intakes (1.5 g per day for adults), and tolerable upper intake levels (2.3 g per day for adults). Some studies are already investigating the possibility of reducing and substituting sodium chloride, as well as the effects of such procedures on sensory quality parameters of different food types [11-15].

The aim of this study was to determine the sodium daily intake through consumption of the most popular meat products (cooked sausages, dry fermented sausages, smoked meat products, canned meats and pates) available on the Serbian market. Also estimation of the potential health risk delivered



from consumption of these products could suggest the necessity for partial reduction of sodium addition and/or substitution of sodium with other salt types.

## 2. Materials and Methods

A total of 163 meat products were collected from the Serbian market (n=53, cooked sausages; n=33, dry fermented sausages; n=44, smoked meat products; n=27, canned meats; and n=6, pates). An amount (0.5 g) of previously homogenized meat was transferred into a microwave digestion teflon vessel along with 5 mL of nitric acid (67% Trace Metal Grade, Fisher Scientific, Bishop, UK) and 1.5 mL of hydrogen peroxide (30% analytical grade, Sigma-Aldrich, St. Louis, MA, USA). The microwave oven (Start D, Milestone, Sorisole, Italy) was set to the following temperature program: 5 min from room temperature to 180°C, 10 min hold at 180°C, 20 min ventilation. After cooling to room temperature, the solutions were quantitatively transferred into polypropylene volumetric flasks and diluted to 100 mL with deionized water obtained from a water purification system (Purelab DV35, ELGA, Buckinghamshire, UK).

Analysis of the  $^{23}\text{Na}$  was performed by inductively coupled plasma mass spectrometry (ICP-MS), (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany). A five-point calibration curve (including zero) was constructed for quantification. Multielemental internal standard ( $^6\text{Li}$ ,  $^{45}\text{Sc}$  – 10 ng/mL;  $^{71}\text{Ga}$ ,  $^{89}\text{Y}$ ,  $^{209}\text{Bi}$  – 2 ng/mL) was introduced along with the sample by an additional line through the peristaltic pump.

The quality of the analytical process was verified by analysis of the certified reference material ERM – BB384 (lyophilized pork muscle; Geel, Belgium). Reference material was prepared in the same manner as regular meat samples. Replicate analyses were in the range of certified values.

Since a total diet study has not been undertaken in Serbia so far, we used the only data available to us, from the Republic Institute for Statistics of Serbia [16], for the purpose of intake assessment. According to this source, estimated average daily consumption of meat product is 54.7 g. The following formula was used for calculation of intake assessment expressed as daily intake (g):

$$\text{Daily intake} = \text{Concentration of Na} \times \text{Daily consumption data}$$

## 3. Results and Discussion

Levels of sodium in different meat products (cooked sausages, dry fermented sausages, smoked meat products, canned meats and pates) from Serbian market are shown in Table 1. Results are reported as mean  $\pm$  standard deviation

**Table 1.** Mean levels of sodium in different meat products

Meat product	Na, g/kg
Cooked sausages	10.4 $\pm$ 2.20 <sup>b</sup>
Dry fermented sausages	16.9 $\pm$ 2.90 <sup>a</sup>
Smoked meat products	11.6 $\pm$ 4.50 <sup>b</sup>
Canned meats	10.8 $\pm$ 2.40 <sup>b</sup>
Pate	4.7 $\pm$ 0.50 <sup>c</sup>

<sup>a-c</sup>Different superscripts indicate significant differences of means according to Tukey's HSD test ( $p < 0.05$ )

Statistically significantly higher levels of sodium were established in dry fermented sausages and lower levels in pate compared with other meat products. Statistical analysis showed there were no significant differences in sodium content between cooked sausages, smoked meat products and canned meats.

The measured sodium level in cooked sausages (10.4 g/kg) was higher than established data in a Finnish study (6.0-9.0 g/kg, [5]) but lower compared with data reported by DTU (18.7 g/kg, [17

Smoked meat products and dry fermented sausages had lower sodium contents than those reported by DTU (13.2-23.0 and 15.0 g/kg, respectively [17]). The determined mean sodium level in canned meats was higher compared with data reported by DTU (7.2 g/kg) [17] and Dietitians of Canada (9.15 g/kg) [18]. Sodium levels obtained in pate (4.70 g/kg) were comparable with data reported by Dietitians of Canada (4.45 g/kg) [18] and lower than the reported level from USDA (6.97 g/kg) [19].

The mean sodium level of all five types of meat products was 11.8 g/kg. According to the average intake of meat products in Serbia (54.7 g/day, [16]) and the measured Na levels, consuming these meat products could contribute up to 26.9% of Na daily requirements (6 g NaCl, i.e. 2.4 g Na, based on national legislation [20]).

#### 4. Conclusion

Having in mind that intake of meat products comprise only 2.76% of total daily food intake, 26.9% of the recommended daily intake of sodium could pose a risk to human health due to the likely significant intake of high levels of sodium through food with a high salt levels, e.g. packaged and processed foods (breads, cereals, cheese, some canned foods, sauces, etc.). Also, humans habitually add salt to food because it improves the positive sensory properties of food, which is perceived as better tasting food. Considering all these facts and taking into account the negative health effects of high levels of sodium, it is necessary to reduce salt levels in meat products and as well as in diets. This could be achieved through partial substitution of sodium salt with other salt types (potassium or ammonium), which is an important topic in scientific literature.

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## Effect of biopolymer coating on texture characteristics of dry fermented sausage during storage

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**Abstract.** In this paper, the changes of texture characteristics of *Petrovská klobása*, a traditional dry-cured sausage, were analysed during 150 days of storage. Sausages were produced in the traditional manner, but underwent drying and ripening processes in industrial conditions. Chitosan was used to coat experimental sausages after the drying process was considered finished. Changes in moisture content were measured during the storage period, but the differences between control and chitosan coated sausages were not significant ( $P>0.05$ ). Changes in moisture content influenced progressive increases in hardness and chewiness of both groups of sausages. The differences in texture characteristics between the different sausage groups were not significant for the entire storage period. Chitosan coating had no positive influence on preventing texture changes in *Petrovská klobása* during storage.

### 1. Introduction

*Petrovská klobása* is a traditional dry-cured sausage made in Vojvodina (the province in Northern Serbia). It is produced in the traditional way, according to original recipes without the use of nitrate/nitrite, gluconolactone (GDL) or microbial starters, in village households at the end of November and during December, when atmospheric temperatures are around 0°C or lower. *Petrovská klobása* is prepared by mixing ground pork meat (the most valuable meat cuts from mature pigs, containing less water, and having more intensive red colour and firmer consistency than pork from younger animals), lard, red hot paprika powder, salt, crushed garlic, caraway and sugar. Sausages undergo a smoking process using specific kinds of wood, and drying and ripening processes for up to 4 months. At the end of ripening, *Petrovská klobása* is characterised by a specific savoury taste, aromatic and spicy-hot flavour, dark red colour and hard consistency [1–3].

The quality of dry-cured sausages and other meat products is strongly affected by their texture characteristics. Many factors affect the final texture of fermented sausages, including ingredients used, processing parameters, acidification method, drying/ripening conditions, as well as interactions among these factors over an extended period of time [4–6].

In order to meet higher market demands for this type of sausage and provide market supply for a longer period during the year, it is necessary, on one hand, to displace production from small household enterprises to industrial plants, on the other hand, to prolong the sausages' shelf life.





Chitosan, as a semi-natural biopolymer, has been extensively researched for edible film application. Chitosan is approved as an additive in the food industry in many countries, has the ability to form films with good barrier properties for gases, demonstrates antimicrobial and antioxidant properties, tends to exhibit fat and oil resistance, but lacks resistance to water transmission [7]. This could be a drawback for use of chitosan films in direct contact with foods and/or for direct handling. In order to improve hydrophilic biopolymer film resistance to water transmission, different fatty materials were incorporated in the film structure, increasing the film hydrophobicity [8].

The suitability of chitosan film with added hydrophobic component, applied as a coating onto dried and ripened sausages to preserve texture characteristics, for achieving a longer storage period was considered in this paper. Thus, the objective of this study was to determine the effects of chitosan coating with addition of caraway essential oil on texture characteristics of traditional dry fermented *Petrovská klobása* sausage during an extended storage period.

## 2. Materials and method

The sausages were manufactured in a traditional manner from lean pork (80% w/w) and back fat (20% w/w), obtained from Landrace pigs. The animals were farmed in a standard production system with a prolonged fattening period (9-12 months; live weight above 130 kg), and slaughtered in a commercial slaughterhouse according to the routine procedure. Meat and back fat were minced to 10 mm particle size and mixed with red hot paprika powder (2.5 g/100 g), salt (1.8 g/100 g), raw garlic paste (0.2 g/100 g), caraway (0.2 g/100 g), and crystal sugar (0.1 g/100 g), until a homogeneous composition was achieved. The batter was stuffed in collagen casings (500 mm long and 55 mm in diameter) and the processes of smoking, drying and ripening were in an industrial ripening room (temperature  $\approx 15^{\circ}\text{C}$ , and average air RH of 76.1 %). The sausage drying process lasted until the required moisture content ( $<35\%$ ) was achieved (65 days) [9].

Chitosan film forming emulsion was prepared as described in Krkić *et al.* [10]. Chitosan powder (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) was dissolved in acetic acid (1% vol.; Proanalitica, Belgrade, Serbia) to reach chitosan mass per volume ratio of  $10 \text{ kg/m}^3$  and then, caraway essential oil (Herba D.O.O., Belgrade, Serbia) at 1 % (v/v) was added to chitosan solution, together with Tween 20 (0.5 % vol, Superlab, Belgrade, Serbia), in order to obtain the coating emulsion.

After drying, one-half of the manufactured sausages were coated with three layers of coating emulsion, using a sponge brush (assigned as coated sausages). Every layer was left to dry overnight before the next layer was applied. The rest of the sausages were left uncoated (assigned as control sausage). After coating, all sausages were stored in a chamber with controlled temperature and relative humidity,  $15^{\circ}\text{C}$  and 75%, respectively for five months.

All determinations were made on three samples from each sausage group (coated and control) in duplicate. Samples for the analyses were taken after 0, 30, 60 and 150 days of storage.

Moisture content was quantified according to the ISO recommended standards [11]. Texture profile analysis (TPA) was performed as described by Bourne [12], at room temperature, using TA.XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY/Stable Micro Systems, Godalming, UK) equipped with a standard cylindrical plate of 75 mm in diameter. The samples 2 cm thick and 2.54 cm in diameter (cylinders), after discarding the external layer of the sausage, were compressed twice to 50% of their original thickness at a constant test speed of 1 mm/s. The following parameters were determined: hardness (kg), adhesiveness, springiness, cohesiveness and chewiness (kg). Measurements were carried out six times for each sausage sample.

One way (ANOVA), Post-hoc (Duncan's test) was performed using the software package Statistica 12.0 for Windows (Stat Soft, Tulsa, Oklahoma, USA). Differences were considered significant at  $P < 0.05$ .

## 3. Results and Discussion

Changes in moisture content and texture characteristics of sausages during the storage period are shown in Table 1. Moisture content after 65 days of production was lower than 35%, as is demanded

by national legislation for dry fermented sausages [9], so at this time, the drying process was considered finished and the coating was applied (day 0 of storage).

During the storage period, the drying process continued in both groups of sausages, and as expected, changes in moisture content were measured as storage progressed, but the differences between control and chitosan coated sausages were not significant. This is probably due to the chitosan film's high permeability to water vapour. Addition of hydrophobic caraway essential oil at the concentration applied was probably not effective in lowering the high water transmission rate of chitosan film to any great extent [13,14]. This could explain why the differences in moisture content were not significant between control and chitosan coated sausages, i.e. the chitosan film could not protect sausages from moisture loss. The moisture content at the end of the storage period (day 150) in both analysed groups of sausages was ~16%.

**Table 1.** Evolution of texture profile and moisture content of *Petrovská klobása* sausages throughout the storage period

Day of storage	Sausage	Hard. *ns	Adh.	Spr.	Coh.	Chew.	Moisture (%)
0	Control	6.64	-76.49	0.385	0.401	2.66	31.48
30	Control	10.91	-33.32	0.356	0.338	3.73	24.86
	Coated	10.98	-31.11	0.368	0.322	3.56	24.38
60	Control	12.04	-22.95	0.352	0.400	4.73	20.62
	Coated	14.24	-35.29	0.332	0.374	5.31	19.71
150	Control	21.42	-25.41	0.370	0.337	7.18	16.17
	Coated	19.39	-23.01	0.354	0.395	7.46	16.32

ns –no statistically significant differences ( $P < 0.05$ ) were measured between control and coated sausages on any one storage day for any of the factors studied; \*Hard. – Hardness; Adh. – Adhesiveness; Spr. – Springiness; Coh. – Cohesiveness; Chew. – Chewiness

The texture characteristics are influenced by the drying process, as after fermentation, drying is a major factor affecting binding and rheological properties of fermented sausages [5,6]. Hardness and chewiness of both groups of sausages, as expected, increased progressively during the storage period. These changes both in hardness and chewiness could be explained by the changes in moisture content in these sausages during storage, since the correlation factors between hardness and chewiness with moisture content were -0.93 and -0.94, respectively. Significant negative correlations between moisture content and hardness and chewiness were registered previously [5,15]. During the whole storage period, hardness and chewiness differences between sausage groups were not significant ( $P > 0.05$ ). Also, during the storage period, there was no significant effect of chitosan film coating on adhesiveness, springiness and cohesiveness.

Previous results considering application of chitosan film for dry fermented sausage coating suggested that this application can lower the intensity of lipid oxidative changes in dry fermented sausages during storage, and that coated sausage had better sensory properties during storage [10,16]. Unfortunately, this positive influence of chitosan coating on prevention of changes in the texture characteristics of *Petrovská klobása* during storage could not be confirmed.

Further investigation might be directed towards application of chitosan coating with a higher percentage of hydrophobic compound or a combination of different hydrophobic compounds, in order to obtain coating with lower moisture permeability and thus better preservation of sausage from moisture loss and consequently texture deterioration.

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# Assessment of process hygiene in take-away restaurants at gas stations in Serbia

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**Abstract.** The present study was conducted to assess the effectiveness of sanitary procedures on food contact surfaces and food handlers' hands in Serbian take-away restaurants at gas stations. For that purpose, a total of 2518 swabs of food contact surfaces and 709 food handlers' hand swabs were investigated for microbiological parameters of process hygiene. The results showed that 11.60% (292 of 2518) of swabs from food contact surfaces, and 16.50% (117 of 709) of swabs from the food handlers' hands were not compliant with the criteria in the self-control plans of the food business operators. Permanent, on-going training of employees on the proper implementation of sanitation procedures is of essential importance to implement effective good hygiene practices and hazard analysis and critical control point plans.

## 1. Introduction

When eating outside the home, consumers expect to obtain quality food with an acceptable food hygiene level, which reduces the risk for food-borne illness [1]. Food safety is primarily achieved through a preventive approach such as the implementation of a food safety management system based on the principles of Hazard Analysis and Critical Control Point (HACCP) and good hygiene practice (GHP). GHP programs are prerequisites to the implementation of a HACCP system and are essential for the production of safe food [2]. Also, the implementation of HACCP system and GHP are mandatory according to European Union and Serbian Regulations. Despite the legal requirements for the implementation of GHP and HACCP, cross-contamination remains an important causative factor in outbreaks of food-borne disease that occurred in restaurants and highlights the continuing importance of GHP with adequate training of food handlers [1].

Food contact surfaces include food containers, utensils, plates, cooking vessels, cutting boards, slicers, knives, steel pallet knives and spatulas, stainless steel and plastic vessels for food distribution [3]. These surfaces are a major concern for food service facilities in controlling the spread of food-borne pathogens because cross contamination via food contact surfaces has been identified as a significant risk factor [4].

A food handler can be defined as anyone involved in the production or preparation of food at any point in the food chain and can include people repairing, maintaining, cleaning or visiting food preparation areas [5]. Food handlers' hands have been identified as one of the crucial vectors for dissemination of microorganisms [6]. Microorganisms are transferred to the hands in the process of food handling and due to poor personal hygiene, which can result in the hands being contaminated with enteric pathogens [7]. The food handler and their contact with contaminated surfaces are potential causes of cross-contamination and, consequently, outbreaks of food-borne disease [8]. To prevent the spread of infection, people in the food production and food service industries should be well trained and motivated to follow good personal hygiene practices, to use correct hand washing procedures and to follow these procedures while working [9].



The aim of this study was to investigate suitable microbiological parameters of process hygiene and to estimate the effectiveness of sanitary procedures for food contact surfaces and food handlers' hands in Serbian take-away restaurants at gas stations, through a three-year period.

## 2. Materials and Methods

During the three year period (from January 2016 to December 2018), an assessment of the process hygiene was carried out in 24 take-away restaurants at gas stations. Overall, a total of 2518 swabs of food contact surfaces and 709 food handlers' hand swabs were investigated for microbiological parameters of process hygiene (Table 1).

**Table 1.** Number of swabs studied in each year

Year	Food contact surface swabs	Food handlers' hand swabs
2016	834	232
2017	846	242
2018	838	235
<b>Total</b>	<b>2518</b>	<b>709</b>

### 2.1. Swab samples

Swab samples from the food contact surfaces and food handlers' hands were taken after cleaning, washing and disinfection procedures. Sampling was conducted according to the standard method [10]. On the sampling day, swabs were transported to the laboratory in a hand-held refrigerator and analyzed within 24 h. All swabs were analyzed in an accredited laboratory according to SRPS ISO/IEC 17025:2006.

### 2.2. Microbiological examinations

Swab from the food contact surfaces were analyzed for aerobic colony count (ACC) according to SRPS EN ISO 4833-1:2014 [11], *Enterobacteriaceae* (ENT) in line with SRPS ISO 21528-2:2009 [12], coagulase-positive staphylococci (STAPH) according to modified SRPS EN ISO 6888-1:2009 [13], and *Listeria monocytogenes* according to SRPS EN ISO 11290-1:2010 [14]. The swabs from food handlers' hands were tested for ACC [11], ENT [12] and STAPH [13]. Results of the microbiological analyses were expressed as number of bacteria per cm<sup>2</sup> (CFU/cm<sup>2</sup>) and number of bacteria per swab (CFU/swab), for swabs taken from the food contact surfaces and food handlers' hands, respectively.

### 2.3. Evaluation of microbiological results

The assessment of the obtained results of microbiological contamination was carried out in accordance with the limit values set by the self-control plans of the food business operators (Table 2).

**Table 2.** Microbiological criteria in the self-control plans of the food business operators

Microorganisms	Porcelain, glass, smooth metal food contact surfaces	Plastic, wood, stone food contact surfaces	Food handlers' hands
Aerobic colony count	≤ 10 CFU/cm <sup>2</sup>	≤ 30 CFU/cm <sup>2</sup>	≤ 200 CFU/swab
<i>Enterobacteriaceae</i>	≤ 10 CFU/cm <sup>2</sup>	≤ 10 CFU/cm <sup>2</sup>	≤ 10 CFU/swab
Coagulase-positive staphylococci	≤ 10 CFU/cm <sup>2</sup>	≤ 10 CFU/cm <sup>2</sup>	≤ 10 CFU/swab
<i>Listeria monocytogenes</i>	Absence in 100 cm <sup>2</sup>	Absence in 100 cm <sup>2</sup>	/

### 3. Results and discussion

During the three year period, in 24 take-away restaurants at gas stations, 3227 swabs were examined: 2518 from food contact surfaces (cutting boards, slicing machines, knives, refrigerator doors, metal working surfaces) and 709 from food handlers' hands. In 2016, a total of 1066 swabs were examined (834 from food contact surfaces and 232 from food handlers' hands), in 2017 a total of 1088 swabs (846 from food contact surfaces and 242 from food handlers' hands), and in 2018 a total of 1073 swabs (838 from food contact surfaces and 235 from food handlers' hands) were studied (Table 1).

The results showed that 11.60% (292 of 2518) of the swabs from food contact surfaces did not comply with the criteria in the self-control plans of the food business operators. In 2016, 19.42% swabs of food contact surfaces were noncompliant with the set limits in control plans, while in 2017, 10.52%, and in 2018, only 4.89% of swabs were noncompliant (Table 3). These findings are close to those conducted by Legnani *et al.* [15] in 2004 and Garayoa *et al.* [16] in 2014. The reduced percentage of noncompliant swabs in 2018 compared to 2016 suggests training of employees on the proper implementation of sanitation procedures is of essential importance to effective GHP and HACCP.

The main reason for noncompliant swabs from food contact surfaces in all three years was the high percentage of swabs with noncompliant levels of ACC (2016 – 95.68%, 2017 – 87.64%, and 2018 – 95.12% of all noncompliant swabs). During the study, coagulase-positive staphylococci and *Listeria monocytogenes* were not detected in any swab from food contact surfaces.

**Table 3.** Microbiological status of the food contact surfaces

Year	Number of swabs	Noncompliant N	%	Finding	Frequency n	%	Finding	Frequency n	%
2016	834	162	19.42	ACC	155	95.68	ACC + ENT	7	4.32
2017	846	89	10.52	ACC	78	87.64	ACC + ENT	11	12.36
2018	838	41	4.89	ACC	39	95.12	ACC + ENT	2	4.88

The results of microbiological examinations of swabs from the food handlers' hands showed that 16.50% (117 of 709) of swabs were not compliant with the criteria in the self-control plans of the food business operators. The results of food handlers' hand swabs showed similar levels of hygiene in all three years (the percentage of swabs of food handler's hands with noncompliant ACC levels: 2016 – 18.10%, 2017 – 15.70%, and 2018 – 15.74%) (Table 4). Again, in swabs from food handlers' hands, ACC levels (in 94.87% of noncompliant swabs) were the main reason for noncompliant results in all years. Coagulase-positive staphylococci were detected only in one swab in 2016, together with aerobic colony count. This worker whose hands carried coagulase-positive staphylococci was referred to an emergency sanitary examination. Enhanced cleaning, washing, and sanitation of accessories, tools and equipment used by this worker were undertaken.

Noncompliant results, obtained by controlling food handlers' hand hygiene, indicate the food production workers did not pay enough attention to the proper procedures for maintaining hand hygiene [17].

**Table 4.** Microbiological status of the food handlers' hands

Year	Number of swabs	Noncompliant N	%	Finding	Frequency n	%	Finding	Frequency n	%	Finding	Frequency n	%
2016	232	42	18.10	ACC	39	92.86	ACC + ENT	2	4.76	ACC + STAPH	1	2.38
2017	242	38	15.70	ACC	35	92.11	ACC + ENT	3	7.89	/	/	/
2018	235	37	15.74	ACC	37	100.00	ACC + ENT	/	/	/	/	/

#### 4. Conclusion

In order to keep the level of process hygiene at an acceptable level, permanent education of workers is necessary in terms of sanitation procedures of food contact surfaces and food handlers' hands. Additionally, continuous monitoring of food contact surfaces and food handlers' hands in accordance with the self-control plans of the food business operators must be conducted using appropriate microbiological analyses.

#### Acknowledgment

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# Characteristics of textured soy protein products as raw materials in the meat industry

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**Abstract.** Soy protein belongs to the group of whole proteins and is recognised as a high quality plant protein. In the meat industry, soy-based protein products are used as functional additives (emulsifiers, stabilizers) or as good nutritional fillers, i.e. replacement for part of the meat in a recipe. They can be added in dry or in hydrated states (textured soy products). As a result of regular product control conducted for the company *Sojaprotein*, data on protein content and water hydration capacity obtained between 2016 and 2018 for textured soy protein SOPROTEX –N, and textured soy concentrate TRADCON T, both minced products, are presented in this paper.

## 1. Introduction

Soy is a unique plant, in that during 3 to 5 months of growth, it can create the largest amount of protein with the most favourable amino acid composition that is suitable for use in human and animal nutrition. Soy proteins belong to the group of complete or whole proteins. A complete protein or whole protein is a food source of protein that contains an adequate proportion of each of the nine essential amino acids necessary in the human diet [1]. Protein quality, as determined by the protein digestibility-corrected amino acid score (PDCAAS) method, is a measure of a protein's ability to provide adequate levels of essential amino acids for human needs. PDCAAS is calculated using an amino acid profile and the true digestibility of a food protein. Soy protein is recognized as a high quality plant protein, but published protein assessment values can vary based on the soy protein ingredient as well as the reproducibility and accuracy of the testing methods [2]. Among the foodstuffs of plant origin, soy products rich in proteins are becoming more significant, as they are used in the production of various meat dishes, baked goods, confectionery products, vegetarian dishes, beverages based on soy proteins and other important products.

## 2. Soy products applied in the meat industry

The main advantages, and therefore the most important reasons to use soy products in the food industry, especially in meat industry, are as follows:

1. Increasing the overall nutritive and biological value and usability of the final product
2. Improvement of the organoleptic properties of the final product
3. Decreasing cost of production of final products

In the meat industry, the protein-made soy products are used as functional additives (emulsifiers, stabilizers) or as good nutritional fillers (replacement for part of the meat in the recipes), with the





purpose of producing positive effects in terms of improving nutritional and sensory properties and assuring standardization of production for meat products in large-scale production series. These positive effects are achieved through the functional properties of soy protein (such as water binding, swelling, gelling, fat emulsification, etc.), the amino acid composition which is very similar to meat proteins, and the abundance of other nutrients (vitamins and minerals).

Under the applicable Serbian legislation on quality of protein products and mixtures of protein products for food industries, protein products from oil seeds (soy) are divided into the following groups:

1. Full-fat products (flour and grits), with minimum protein content of 38% in dry matter (dm) and fat content minimum 18% dm;
2. Semi-fat products (flour and grits), with minimum protein content of 45% in dm (44% dm for grits) and maximum fat content of 9% in dm;
3. Defatted soy products (flour and grits), with minimum protein content of 47% in dm and maximum fat content of 2% in dm;
4. Concentrated soy protein, with minimum protein content of 65% in dm and maximum fat content of 2% in dm;
5. Isolated soy protein, with minimum protein content of 86% in dm and maximum fat content of 1% in dm.
6. Structural soy proteins

Because of their high oil content (and the unsaturated fatty acids they contain), full-fat and semi-fat soy products are avoided in the meat industry.

Soy products intended for meat industry can be used alone or in combination with other protein products in various meat products, such as different types of sausages (cooked finely chopped, cooked roughly chopped, cooked with meat pieces, cooked, fresh, frying sausages, etc.), shaped minced meats, smoked products, ready meals and canned meats. The use of soy protein products in the meat industry is usually very simple. The soy products can be added in dry or in hydrated states (textured soy products), and thereafter are treated like meat ingredients, so they do not interfere with the technological processes of meat product manufacture.

### **3. Soy protein products from *Sojaprotein***

*Sojaprotein* is the most important and largest soy processor in Central and Eastern Europe, with a processing capacity of over 250,000 tons of soy annually. The company was established in 1977 while in 1983 regular production started, and has continuously been operating since. In 2012, a new section for production of soy concentrate went into operation. Special attention is paid to the quality of raw material and to ensuring that the soy is not genetically modified (non-GMO).

*Sojaprotein* products used in the meat industry include:

- Defatted soy flour;
- Soy protein concentrates;
- Textured soy protein (from soy flour and soy protein concentrates).

SOPROTEX N minced is a textured product mainly used as a filler (to replace meat in a recipe). SOPROTEX N minced expresses its optimal properties when it is hydrated, by adding 2-3 parts of water to one part of flaked product. The usual dosage is 2-4% SOPROTEX N minced, calculated according to the weight of the final product. It can be used in different types of sausages, minced products and shaped meat products.

TRADCON T minced is a textured soy protein concentrate. The production process for traditional soy protein concentrates involves deactivating anti-nutritional factors, thus increasing the usability of the proteins. The removal of part of the soluble carbohydrates results in TRADCON T having a more neutral taste and lighter colour than soy flour-based textured products. TRADCON T minced is also mainly used as a filler. It expresses its optimal properties when it is hydrated, by adding 3-4 parts of water to one part of TRADCON T minced. The usual dosage is 2-4 % of the calculated weight of the

final product. This soy product can be used in different types of sausages, minced products and shaped meat products.

#### 4. Analysis of textured soy protein SOPROTEX-N minced and textured soy concentrate TRADCON T minced

*SP Laboratories* performs regular quality control of the physical and chemical properties, as well as microbiological parameters of *Sojaprotein's* products. This paper presents results of analysis for protein content (% in dm) and water hydration capacity (WHC) for the period between 2016 and 2018, for textured soy protein SOPROTEX-N minced and textured soy concentrate TRADCON T minced. Protein content was determined by the total combustion method [3]. WHC is defined as the maximum amount of water that 1 g of material will imbibe and retain under low speed centrifugation [4].

The percentage of soy products examined during 2016, 2017, and 2018 in various categories of protein content in dm (Tables 1 and 2) and WHC (Tables 3 and 4) are shown. In each category, the percentage of all results that fit the given range is shown.

**Table 1.** Percentage (%) of SOPROTEX-N minced soy samples analysed in 2016-2018, categorised according to percent of protein in dry matter (dm)

Protein content	<50 % protein in dm	50-52 % protein in dm	52-54 % protein in dm	54-55 % protein in dm	> 55 % protein in dm
2016	0.45	1.35	43.24	43.24	11.71
2017	0	0.87	45.02	28.14	25.97
2018	0	1.22	13.88	45.71	39.18

**Table 2.** Percentage (%) of TRADCON T minced soy samples analysed in 2016-2018, categorised according to percent of protein in dry matter (dm)

Protein content	<68 % protein in dm	68-70 % protein in dm	>70 % protein in dm
2016	0	80.00	20.00
2017	0	42.86	57.14
2018	0	62.50	37.5

**Table 3.** Percentage (%) of SOPROTEX-N minced soy samples analysed in 2016-2018, categorised according to water holding capacity

WHC	<2.5 cm <sup>3</sup> /g	2.5-3.0 cm <sup>3</sup> /g	3.0-3.5 cm <sup>3</sup> /g	3.5-4.0 cm <sup>3</sup> /g	>4.0 cm <sup>3</sup> /g
2016	0.71	16.96	63.25	18.2	0.88
2017	0	7.53	63.22	26.59	2.66
2018	0.13	3.79	72.36	22.36	1.36

**Table 4.** Percentage (%) of TRADCON T minced soy samples analysed in 2016-2018, categorised according to water holding capacity

WHC	<4.0 cm <sup>3</sup> /g	4.0-4.5 cm <sup>3</sup> /g	>4.5 cm <sup>3</sup> /g
2016	0	80.00	20.00
2017	0	42.86	57.14
2018	0	62.50	37.5

## 5. Conclusion

The results presented in this paper demonstrate the quality of SOPROTEX-N minced and TRADCON T minced is of a high standard and is temporally stable as far as protein content and WHC are concerned. In 2016, 99.55% of SOPROTEX-N minced samples examined complied with protein content quality standard of a minimum of 50% protein in dm, while in 2017 and 2018, 100% of the SOPROTEX-N samples examined achieved this quality minimum. The WHC was higher than 3cm<sup>3</sup>/g in between 82.33% and 96.08% of samples examined. The protein content in TRADCON T minced was always higher than the minimum 68 % in dm, while the WHC was always higher than 4cm<sup>3</sup>/g.

Because of their high protein content, high water hydration capacity, favourable amino acid composition, and other properties, soy protein products have found widespread application in the meat industry. Soy products, when used in meat products, show positive effects in terms of improving the sensory properties, while ensuring stable production as well as efficiency for large-scale production series. Although products made from meat with added soy protein products mostly cost less than products without soy, consumers get a measurably (organoleptic, nutritional, etc.) better product, and are not damaged in any respect. Disclosure of soy protein on the product package is, however, mandatory, in order not to mislead consumers in terms of the contents and quality of the products they purchase, and in order that manufacturers do not attain ungrounded financial profits.

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# The influence of the basil on colour, odour and taste of frankfurters

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**Abstract.** Plants from the moderate climate zone are rarely used as seasoning in pasteurised sausages. Seasoning plants of the family *Lamiaceae* belong to this group (marjoram, basil, savory, sage, thyme). One of the reasons these herbs are rarely used to season meat products is the presence of the plant pigment, chlorophyll that can have undesirable influence on colour, odour and taste of meat products. The aim of this paper was to assess the influence of basil on colour, odour and taste of vacuum-packed frankfurters during 28 days of storage. Powdered, dried basil (0.1%, 0.3% and 0.5%) was added to frankfurters while control frankfurters were produced without herbs or spices. Assessment of colour, odour and taste acceptability of the frankfurters was performed by a panel of five assessors using a quantitative descriptive test, and the results of the ranking test were analysed statistically. When used at the level of 0.1% in the sausages, basil did not have negative influence on frankfurter colour, while larger quantities 0.3% and 0.5% adversely affected frankfurter colour. Basil in the smaller amounts of 0.1% and 0.3% stimulated development of a pleasant odour and taste in the frankfurters, while 0.5% basil had an undesirable effect on these sensory attributes.

## 1. Introduction

In the production of meat products, tropical spices rich in essential oils are most commonly used. Herbal plants from moderate climate zones, of which the leaves and parts of the stem plants are used, contain slightly less essential oil than tropical spices. Herbs of the family *Lamiaceae* (basil, savory, sage, thyme, marjoram, etc.) that contain essential oils and other active substances are grown in Serbia. Dried and finely chopped, these plants have intensive aromatic odours and similar colours – green to grey-green. Their taste ranges from slightly to strongly “burning”, and some of them have a bitter taste. One of the reasons these herbs are uncommon as seasonings in meat products is their content of the plant pigment chlorophyll, which can have undesirable influences on colour, odour and taste of the meat products [1].

Basil (*Ocimum basilicum* L.) has a pleasant, aromatic odour. The flavour is aromatic, salty and warm, with a bitter taste. Carriers of the characteristic odour and taste of basil are essential oils, tannins, flavonoids, saponins, etc. Basil contains from 0.3% to 0.8% essential oil, of which the main ingredients are carvacrol and linalool [2]. In Mediterranean countries, basil is used for making sauces, marinades, tomato dishes and for seasoning sheep meat and pork, etc.



In Germany, sausages made with herbs from the family *Lamiaceae* (basil, thyme, marjoram) are called “*Kräuterwurst*”. Basil and thyme are most often used in the production of cooked sausages, rarely in pasteurised sausages, and are the least common in fermented dried sausages. Basil (0.01%) is added to two types of liver sausage, while basil and thyme (0.5%) are added to one type of pasteurised sausage [3].

According to the literature data and our knowledge of current industry practice, basil is very little used in the Serbian meat industry, especially in pasteurised sausages. The aim of this study was to assess the influence of basil on colour, odour and taste of vacuum-packed frankfurters during 28 days of storage.

## 2. Materials and methods

### 2.1. Raw material composition

Frankfurters were produced from beef meat (50%), pork fat (25%) and ice (25%). To 1 kg of stuffing, 18 g of nitrite salts and 3 g of polyphosphate were added. Powdered, dried basil was added to the experimental frankfurters (0.1%, 0.3% and 0.5%), while the control frankfurters were produced without any herbs or spices. The frankfurter stuffing was filled in artificial cellulose casings. Frankfurters were thermally processed (72°C in the centre of the product) and then cooled. After cooling, frankfurters were vacuum packed. All packages of frankfurters were stored in the same conditions at 4 °C and on days 1, 7, 14, 21 and 28 of storage, sensory testing was performed.

### 2.2. Chemical analysis

The content of essential oils in the dried herb was analysed according to [4].

### 2.3. Sensory analysis

Sensory evaluations were performed by five trained panellists. Frankfurter colour was analysed using a quantitative descriptive test [5], with grading scale from one to five (1 – unacceptable colour; 2 – very low level of acceptability of colour ; 3 – acceptable colour; 4 – good colour; 5 – exceptionally good colour).

Using quantitative descriptive test [5], with grading scale from one to seven, the frankfurters' sensory properties of odour and taste were analysed (1 – extremely unpleasant odour and taste; 2 – unpleasant odour and taste; 3 – unpleasant odour and taste; 4 – neutral odour and taste; 5 – pleasant odour and taste; 6 – very pleasant odour and taste; 7 – exceptionally pleasant odour and taste).

### 2.4. Statistical analysis

Results of the sensory evaluation ranking tests [6] were analysed statistically [7].

## 3. Results and discussion

The dried basil used in the frankfurters contained essential oil (0.8ml/100g), a level in accordance with legal regulation in Serbia [8].

**Table 1.** Sensory evaluation of the colour of vacuum-packed frankfurters during storage

Day	Percentage of basil in frankfurter	Sum of ranks	Differences in frankfurter colour according to percentage of basil		
			0	0.1	0.3
1	0	6			
	0.1	14	8		
	0.3	16.5	10.5	2.5	
	0.5	23.5	17.5**	9.5	7
7	0	6			
	0.1	12	6		
	0.3	19.5	13.5*	7.5	

<b>14</b>	0.5	22.5	16.5**	10.5	3
	0	6			
	0.1	12	6		
	0.3	20	14**	8	
<b>21</b>	0.5	22	16**	10	8
	0	6			
	0.1	13.5	7.5		
	0.3	17	11	3.5	
<b>28</b>	0.5	23.5	17.5**	10	6.5
	0	6.5			
	0.1	12	5.5		
	0.3	19.5	13*	7.5	
	0.5	22	15.5**	10	2.5

\*  $p \leq 0.05$  – statistically significant difference; \*\* $p \leq 0.01$ ) – highly statistically significant difference

There was a statistically significant difference ( $p < 0.05$ ) between the colour of control frankfurters and frankfurters with 0.3% basil on days 7 and 28 of storage. The differences in colour on day 14 of storage were highly statistically significant ( $p < 0.01$ ).

During storage, statistically significant differences ( $p < 0.01$ ) were observed between the colour of control frankfurters and frankfurters with 0.5% basil. No statistically significant differences were detected between the other frankfurters studied.

**Table 2.** Sensory evaluation of the odour and taste of vacuum-packed frankfurters during storage

Day	Percentage of basil in frankfurters	Sum of ranks	Differences in frankfurter odour and taste according to percentage of basil		
			0	0.1	0.3
<b>1</b>	0	12			
	0.1	10.5	1.5		
	0.3	17.5	4.5	7	
	0.5	20	8	9.5	2.5
<b>7</b>	0	7			
	0.1	11	4		
	0.3	18.5	11.5	7.5	
	0.5	23.5	16.5**	12.5*	5
<b>14</b>	0	17.5			
	0.1	9	8.5		
	0.3	16	1.5	7	
	0.5	17.5	0	8.8	1.5
<b>21</b>	0	16			
	0.1	8	8		
	0.3	14.5	1.5	6.5	
	0.5	21.5	5.5	13.5*	7
<b>28</b>	0	24			
	0.1	10	14**		
	0.3	13	11	3	
	0.5	13	11	3	0

\*  $p \leq 0.05$  – statistically significant difference; \*\* $p \leq 0.01$ ) – highly statistically significant difference

The odour and taste of frankfurters with 0.1% basil and frankfurters with 0.5% basil were statistically significantly different ( $p < 0.05$ ) on days 7 and 21 of storage. On day 7, a statistically significant difference ( $p < 0.01$ ) between the odour and taste of control frankfurters and those with 0.1% and 0.5% basil was detected. On day 28, the odour and taste of control frankfurters and the odour and taste of frankfurters with 0.1% basil were statistically significantly different ( $p < 0.01$ ). No statistically significant differences were detected between other frankfurters on this day.

There is little data in the literature of the use of basil in pasteurised sausages. However, the results obtained are in accordance with [9], where basil (at different concentrations) was added to dry fermented sausage. Basil 0.1% did negatively influence the colour of dry fermented sausage, while 0.3% and 0.5% basil had a negative influence. According to the same author [9], basil at the levels of 0.1% and 0.3% stimulated development of a pleasant odour and taste in dry fermented sausage, while 0.5% basil had an undesirable effect. According to other authors [10], basil has a pleasant odour and taste, but more than 0.03% cannot be added to cooked sausages. Basil causes off-taste in poultry meat products that are thermally treated by pasteurization or sterilization processes [11].

#### 4. Conclusion

Based on its content of essential oil, the basil used was of good quality, and was characteristic of the climate in Serbia. Basil 0.1% did not negatively influence the colour of frankfurters, while larger amounts 0.3% and 0.5% did adversely affect frankfurter colour. Basil at levels of 0.1% and 0.3% stimulated development of pleasant odour and taste of frankfurters, while 0.5% basil had an undesirable effect. In conclusion, frankfurter with 0.1% basil had the most desirable sensory attributes. The results show that there is a real possibility of using basil in spice mixtures for the production of frankfurters.

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# Partly processed meat products prepared for grilling as a source of protein

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**Abstract.** In the period January 2018 to December 2018, the levels of protein were measured in 864 samples of partly processed meat products prepared for grilling, from the Serbian market. Just 2.89% of these partly processed meat products contained protein content less than 14 g/100g, and so did not meet the requirement laid down in the national regulation. By groups, 97.89% of minced shaped meat, 98.71% of minced unshaped meat and 93.62% of fresh sausages met the requirement of national regulation. The meat product group with the biggest percentage of non-compliant meat products was the fresh sausages group – 6.38% of these contained less than the required protein content. All in all, most of the partly processed meat products met the national regulation regarding protein content. With average  $16.23 \pm 1.82$  g/100g of protein in raw, pre-thermal treatment samples, this group of meat products would be a good source of protein in human nutrition. Nonetheless, those meat products that did not meet national regulation requirements show that regular and periodic control of partly processed meat products is necessary and one of the most important steps in ensuring safe and quality meat products for consumers.

## 1. Introduction

Protein is an indispensable nutrient that plays many critical roles in the human body including building muscles, tissue cells, making hormones and antibodies, transporting substances into and out of cells, controlling chemical reactions, transmitting messages from one part of the body to another, and much more.

A protein molecule is made of a long chain of amino acids. There are 20 types of amino acids in proteins, each with different chemical properties [1]. Meat and meat products are one of the most important sources of essential amino acids in human nutrition and provide protein of high biological value. Conventional animal-based protein sources such as meat (beef, pork, lamb, poultry, etc.), fish, eggs, and dairy are generally considered high-quality sources of dietary protein because they meet all of the indispensable amino acid requirements for humans and are highly digestible [2]. Beside this, red meat is the best source of iron, unlike pork and chicken, which contain moderate amounts of iron. That is why vegetarians have a higher risk of developing iron deficiency, as iron obtained from plants has a lower bioavailability, so they often have to take supplements in order to compensate for the lack of iron and essential amino acids. Nevertheless, human population studies show that red and processed meats are associated with an increased risk of colorectal cancer, while white meat has no impact on this type of disease. The evidence is not yet conclusive, but consuming a lot of preserved meat probably increases the risk of colorectal cancer [3,4,5]. Some studies showed this risk can be lowered





with addition of fiber from different sources [6,7]. Back to the protein, the current international recommended dietary allowance (RDA) for protein is 0.8 g per kg of body weight, regardless of age [8,9]. According to the FAO, human requirements for protein are currently estimated to be 55 g per day for adult men and 45 g for women, although there is a higher requirement in various disease states and conditions of stress. These amounts refer to protein of what is termed “good quality” and highly digestible, otherwise the amount ingested has to be increased proportionally to compensate for lower quality and lower digestibility [10]. In Serbia, national regulation [11] states that 50 g of protein per day is the recommended amount for adults. Another national regulation [12] requires that samples from partly processed meat products group must contain at least 14 g of protein per 100 g of meat product. “Pljeskavica” (Serbian-style meat patties of various types) and “ćevapčići” (grill kebabs), must contain a minimum 14% of meat protein. On the other hand, all other products (e.g. hamburger, burger, ćevapi, etc.) can contain, beside the already present meat protein, other sources of substitute protein, i.e. soya and other. The total protein content in this case also has to be at least 14 g per 100 g sample.

Meat composition, as well as its physicochemical properties, undergoes significant changes during heat treatment. It is well known that meat composition, especially its fat content combined with the specific cooking methodology, is among the factors that most affect the final quality of meat products [13]. This also stands for other macronutrients, including protein, and after water loss during thermal treatment, protein concentration in product raises. However, this will not be part of this scientific paper, as testing was conducted just in raw samples, before thermal treatment.

The aim of this study was to determine levels of protein in retail partly processed meat products, before thermal treatment, commonly consumed in Serbia.

## 2. Materials and Methods

### 2.1. Partly processed meat products

In the period January 2018 to December 2018, 864 samples of different types of partly processed meat products prepared for grilling (521 shaped minced meat, 155 unshaped minced meat and 188 fresh sausages) were tested for protein content. Samples were from the Serbian market and were produced by local producers. Testing was conducted in raw samples, before thermal treatment.

### 2.2. Method of chemical testing

The protein content was determined according to the standard procedure [14], expressed as percentage (g/100g), and measured by FOSS Kjeltac™ 8400 Analyzer. All chemicals used for testing the protein content in the partly processed meat products prepared for grilling were of analytical grade and were used as received without any further purification.

### 2.3. Statistical analysis

The results analysis and graphical presentation of their distribution was performed using Microsoft Office Excel 2016.

## 3. Results and Discussion

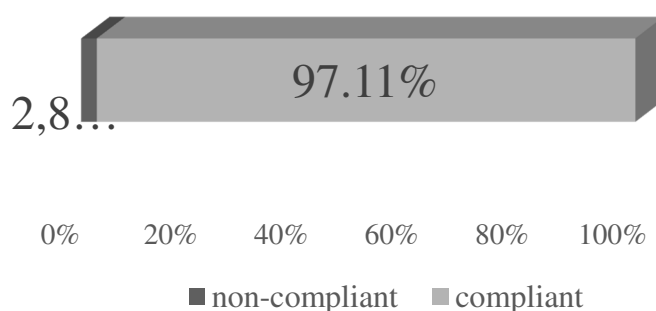
Protein contents of the grouped meat products are shown in Table 1. The distributions of the results, in accordance with the national regulation [12] and its requirement for minimum protein content (14.00%), are graphically presented in Figure 1 for all samples.

**Table 1.** Protein content in samples of partly processed meat products prepared for grilling, for the period January 2018 – December 2018

Type of product	n <sup>a</sup>	n <sup>a</sup> (%)	Min	Max	Xs <sup>b</sup> ±S <sub>d</sub> <sup>c</sup>	CV <sup>d</sup> (%)	n <sup>e</sup>	n <sup>e</sup> (%)	n <sup>f</sup>	n <sup>f</sup> (%)
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Minced shaped meat	521	60.30	9.17	21.83	16.33±1.76	10.78	11	2.11	346	66.41
Minced unshaped meat	155	17.94	10.09	21.35	16.33±1.62	9.92	2	1.29	60	38.71
Sausages	188	21.76	8.86	22.34	15.89±2.09	13.15	12	6.38	120	63.83
All samples	864	100.00	8.86	22.34	16.23±1.82	11.21	25	2.89	526	60.88

<sup>a</sup> number or percentage of samples; <sup>b</sup> average value; <sup>c</sup> standard deviation; <sup>d</sup> coefficient of variation; <sup>e</sup> number or percentage of non-compliant samples; <sup>f</sup> number or percentage of samples which contain just meat protein



**Figure 1.** Percentage of all tested samples in accordance with the national regulation [12] regarding protein content

Most of the tested meat samples, 97.11% of them, met the national regulation [12] regarding the minimum content of protein. The minimum (8.86%) and maximum (22.34%) concentrations of protein were determined in the group of fresh sausages. This group of meats had the smallest average protein content (15.89%), and the highest percentage (6.38%) of tested samples that did not meet the national regulation [12] requirement of minimum 14% protein content. Minced unshaped meat had the lowest percentage of samples that did not meet the same requirement, 1.29% of them. The maximum average protein content (16.33%) was determined as the same in both groups of minced meats (shaped and unshaped) but there was no big difference between the average protein contents of any of the different tested groups. Minced shaped meats produced the highest percentage of samples containing just meat protein (66.41%), and minced unshaped meats produced the lowest, just 38.71% of samples from this group. The most common meat product type sampled for this study was the group of minced shaped meats (n=521, or 60.30% of all meats sampled).

#### 4. Conclusion

The majority of raw meat products tested in this research, 97.11% of them, across the selected meat product groups, met the national regulation [12] requirement for minimum protein content. Over 60% of samples contained only meat protein, without any protein substitutes, i.e. soya or other protein. Requirements [8,9,10,11] for protein intake are around 50 g/day, and the average content of protein in the tested raw meat products was 16.23 g/100g, but after thermal treatment of raw product, the concentration of protein should be higher because of water content loss. According to these results, it is clear that partly processed meat products prepared for grilling are a good source of protein in human nutrition.

Aside from protein content, in the meat products tested, we also determined the content of other quality parameters, i.e. collagen, but this is not reported here. The small number of partly processed meat products that did not meet the national regulation [12] requirement leads us to conclude that periodic and regular control by the responsible authority is a necessary step in ensuring quality and safety of meat products and food in general.

## Acknowledgement

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# Systemic risk analysis of complex meat systems

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**Abstract.** The principles of the Hazard Analysis Critical Control Points (HACCP) system focus on the risks to product safety. For complex meat systems with the longest shelf life (canned meat in pieces with up to 5 years' shelf life), the problem of quality assurance using suitably stable safety indicators throughout their entire shelf life is systemic. We propose to use the methodology of a systemic approach for solving this problem. The general signs of systemic problems are given, and their contents are described, largely in the context of canned meat pieces. An example of the use of structural analysis diagrams (see D. Ross) to analyze quality assurance and product safety is shown. The relevance of systemic analysis methodology for finding solutions to practical problems is explained.

## 1. Introduction

Any production activity is accompanied by foreseen and unforeseen risks. The issue of the production organization with the timely identification of risks, their analysis and decision-making related to them is a key aspect of the release of safe and competitive products to the market.

The process of canned meat production is a complex system with diverse connections, characterized by continuity and discreteness, determinism and randomness. Besides this complexity, canned food, during its entire shelf life, should be considered as a complex system, experiencing some fluctuations. Under the influence of specific factors, these fluctuations can increase and loosen the links between the elements of the system. Such instability over time, if at a critical level, could lead to rapid changes in the macroscopic behavior of a complex system. It is also possible that behavior becomes more coherent (the links tighten) when a new interaction occurs between the elements of the system [1].

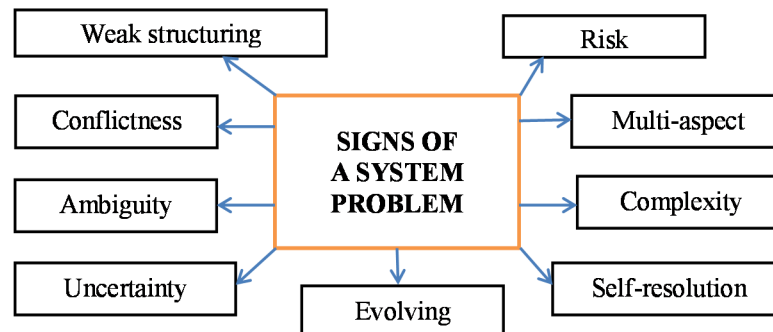
It is impossible to establish a connection between invisible, quantitative changes at the micro-level and visible qualitative changes at the macro-level of complex systems, or to determine the critical value of a parameter from purely abstract, theoretical considerations. The additive models (dependencies) normally used do not always agree with the physical sense of the task. There is the question of interactions of components, and as a consequence, the food industry pays inadequate attention to investigation of processes occurring when such interactions occur. Investigative scientific systems still have a limited array of methods and tools to study the properties of the complex food systems in question. In the majority of cases, an adequate theoretical base for describing the physical, chemical and biological properties of food systems is lacking. Altogether, these complexities lead to problems in managing system's conditions and technological processes. However, systemic analysis allows us to utilize quantitative and qualitative categories to investigate a problem in complex food



systems, specifically, producing canned meat pieces with long shelf life duration but with suitable safety and quality indicators.

## 2. Identification of a systemic problem

The concept of a systemic problem is one of the central ideas in modern theory of systemic analysis [2]. Identification of a problem as a systemic one can be based on the analyzing of the main features (Figure 1).



**Figure 1.** Signs of a systemic problem

With regard to complex systems such as canned meat, each feature has its own objective orientation.

*Unstructured or weak structuring.* According to Simon's classification [3], this feature of the problem is the basis of system analysis, but it does not reveal the internal content of the problem and is mainly qualitative in its formulation and description of the problem. In relation to canned food a combination of quantitative assessments are used with indicators of quality and safety.

However, qualitative parameters with some uncertainties tend to dominate at the beginning of the process. The lack of uniformity in the quality of meat is a common problem of the meat processing industry, because fluctuations in the quality of meat lead to fluctuations in the quality of the finished product [4, 5].

Today in the canning industry, sorting of meat according to maturation defects is carried out only in the production of canned ham. *A priori*, it is believed that the meat arriving for processing is properly matured. As a result, there is a high risk of obtaining finished canned meat products with impaired organoleptic characteristics, such as the stratification of a supposedly homogeneous pâté or unacceptably tough pieces of meat in canned meat pieces.

From the point of view of quality and safety, dark, firm dry (DFD) meat is problematic to store because it is subject to rapid microbial spoilage [6-9]. Production of sterilized products from such meat will neutralize the microbiological risks, but technological risks will come to the fore. For example, the consistency of the meat after sterilization will be different, with fibrillation of muscle fibers, lack of juiciness, and lack of typical, pronounced taste and aroma, which is unacceptable to the consumer.

The dominant factor in ensuring the safety of canned food is the level of contamination of the main raw materials and ingredients with spore-forming microorganisms. However, in the production of canned food, especially when choosing sterilization modes, there is no online algorithm for the dependence of sterilization modes on the spore load of raw materials and ingredients, alone or in mixtures, before heat treatment, which corresponds to the essence of the sign of "conflictness".

*Conflictness* is the contradiction between the immanent desire for a particular goal and the limited possibilities for the practical realization of this aspiration. In canned food production, this conflictness is illustrated by the need to obtain safe canned food with the specified quality indicators for the

consumer, on the one hand, and the need to limit the product's exposure to high temperatures and pressure in the sterilization process on the other. Therefore, the transfer of the canning industry to less stringent sterilization regimes [10] in the complete absence or fragmentation of microbiological standards for meat raw materials, ingredients and their mixtures before heat treatment limits the possibility of practically realizing this aspiration. The solution to this problem will be to find compromise solutions between the multifactorial nature of this complex meat system and the multiplicity of criteria for assessing its quality and safety.

The uncertainty lies in the fact that it is impossible to take into account in advance all the situations that will have to be faced in addressing the quality and safety of sterilized canned food. Primarily, this requires decoding the mechanisms of intramolecular and intermolecular interactions of protein and fat components of canned food during sterilization. The solution to this problem involves the revision of traditional technological solutions, using innovative methods and methodological approaches. Not unimportant is the microbial component of the canned food before sterilization. It is impossible to accurately forecast the lethal effect of thermal loads and further behavior of the residual microbiota of canned food during storage due numerous intrinsic factors, such as pH, water activity, the presence of antimicrobial components, etc., which can change the behavior of the microbiota [11]. The use of rapid methods and mathematical models for predicting the behavior of the microbiota during the technological processing, starting with input control [12-14], will remove some uncertainty on this issue.

*Ambiguity.* Systemic problems can be solved in several ways. The choice of these options is made on the basis of scientific knowledge and intuition. According to Novoseltsev [2] "intuition, supported by knowledge, and scientific creativity play a significant, and sometimes decisive role in the system analysis, being the source of new ideas and ways to resolve systemic contradictions."

An example of existing ambiguity in canned food processing is the outdated methodological approach to the development of sterilization regimes. Firstly in the current approach, vegetative and spore forms of microorganisms are placed directly inside the consumer packaging (the can) with the food, and the survival of the microorganisms is measured by submitting the food to the production process. Secondly, sterilization regimes are then based on the worst-case scenario of the microbial component of the prescribed ingredients, resulting in the applied sterilization regimes being excessively rigid.

A change in methodological approach is needed, to develop optimum and rational sterilization modes through the use of innovative test systems that will support development of appropriate modes of sterilization, without risking microbial spoilage developing during can storage. Additionally, systematic compliance monitoring of the temperatures of the heating medium in the autoclave and contents in the consumer packaging (the can) during the sterilization process will ensure the stability of safety and quality indicators in the food.

*Risk.* To solve a systemic problem, resource investments are necessary, and any investments are accompanied by risks. If risks are not prevented at the initial stage of production, for example, raw meat with defects or that does not meet hygienic standards is used, and are unable to be taken into account in the future, then this set of risks will lead to a product that does not meet the specified safety and quality indicators [15]. In addition, microbiological risks lie in the current absence of control over toxins of microbial origin in raw materials, ingredients and finished products.

*Multi-aspect.* This area of analysis of the systemic problem of obtaining a safe product of high quality is associated with the issues of production, technological methods, economic features, and the set of methods used to assess the physico-chemical, biochemical, histological, microbiological, and organoleptic characteristics of the finished product.

*Complexity.* This area focuses on achieving the goal by utilizing a set of results of independent research (organoleptic, biochemical, microbiological, etc.) together with the use of innovative methods. Together, this complex data must reliably reflect the multidimensional nature of the quality variability of complex meat systems and aim at understanding the essence of the processes.

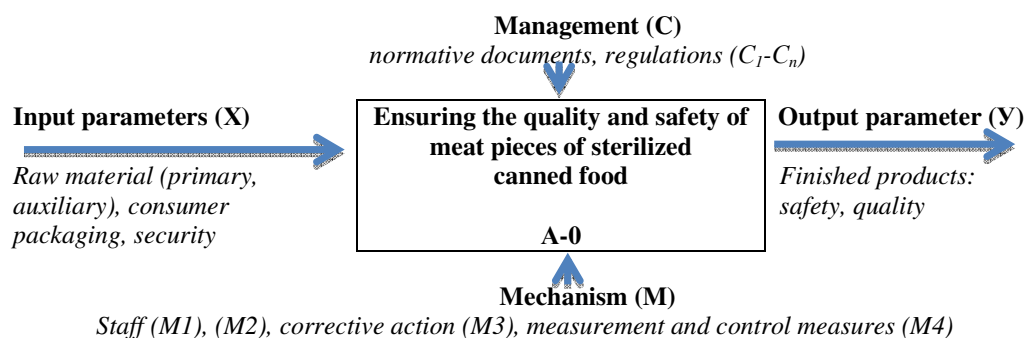
*Self-resolution.* The problem can resolve itself only in the presence of optimal management and using technological solutions that do not provoke negative, deleterious outcomes for the canned product at the micro- and macro-levels. Self-resolution should also contribute to the negative dynamics of inactivation of heat-resistant spores during sterilization, and only products with satisfactory safety and quality indicators should result.

*Evolving.* The problem of ensuring the safety and quality of sterilized canned meat is not a new problem. Trying to solve the problem of canned food quality specifically by reducing the temperature load creates problems within the framework of safety, and *vice versa*, focusing on microbiological safety means the quality of the canned meat deteriorates [16-18]. This process is not only not interrupted, but can be branched. This systemic production problem in canned food must be solved taking into account the continuum of knowledge *re* mechanisms of intra- and inter-molecular interactions of protein and fat components in canned food during sterilization, the survival of spores and toxin-forming microorganisms, and the heat resistance of spores and toxins. This should eliminate the emergence of new, even more unsolvable problems, such as the formation of toxins and heat-resistant strains of microorganisms surviving during the storage of the canned meat [19].

In general, in analyzing the signs of the systemic problem of ensuring the safety and quality of sterilized canned meat, it is clear that any problem that occurs throughout the shelf life of this food product is always systemic in nature and can be solved on the basis of a systematic approach that reflects its diversity. Production of sterilized canned meat with the required stable quality and safety characteristics is connected with specific risks that interfere with successful production. It is necessary to identify microbiological and technological risks arising in the production and storage of canned food, to assess them and identify management decisions to mitigate them. Using the principles of systemic analysis, possible sources of hazards and product defects are necessarily structured for a surer, positive outcome.

### 3. Applying systemic analysis to production of canned meat pieces

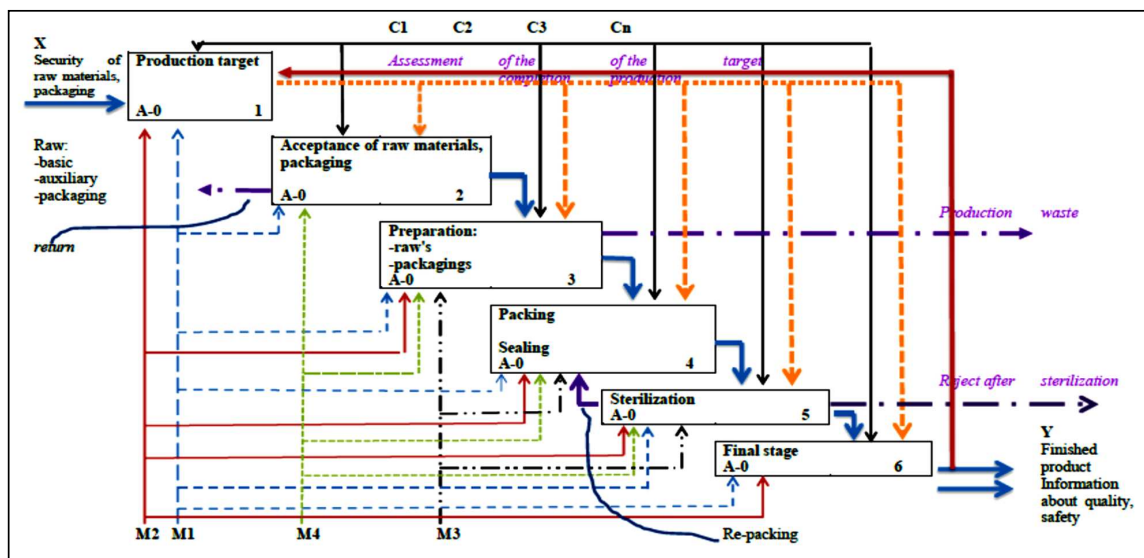
Using the methodology of system analysis as applied to canned meat production, we have developed a context diagram of the upper level (A minus zero), presented in Figure 2, showing a set of hierarchical actions to ensure the quality and safety of canned meat pieces.



**Figure 2.** General model A-0 – identification and assessment of microbiological and technological risks in ensuring the quality and safety of canned meat pieces.

The only function is to ensure the safety and quality of sterilized, canned meat pieces. The top-level context diagram (Figure 2) has been decomposed into the main sub-functions by creating a subsidiary diagram (Figure 3) showing detailed analysis of the risks arising in the various stages of canned meat production.





**Figure 3.** Decomposition Of A-0

As can be seen from Figure 3, the process of canned food production consists of six blocks:

- i) block 1 – Production task;
- ii) block 2 – Acceptance of raw materials and consumer packaging;
- iii) block 3 – Preliminary preparation of raw materials and consumer packaging;
- iv) block 4 – Packing the contents of canned food in consumer packaging and its sealing;
- v) block 5 – Sterilization of canned food;
- vi) block 6 – Final stages.

Each block describes a definitive aspect of the process and can be represented as a set of interrelated factors, together describing in detail the specific process of the block.

Operations block 1 is carried out in accordance with the regulations on the production of canned food (C1-Cn). Personnel and services (M1) responsible for the preparation of initial information (X) based on the production capabilities of the meat processing enterprise are considered as the mechanisms for the implementation of processes.

One of the determining blocks in the formation of quality and safety of finished canned products is block 2. Operations in this unit are carried out in accordance with regulations and norms (C1- Cn), reflecting the requirements and methods of research. The main and auxiliary raw materials and the consumer packaging are subjected to entrance acceptability quality assurance using control and measuring actions (M4), and materials will be accepted from suppliers if their normalized microbiological and biochemical indicators, organoleptic characteristics and hygienic indicators of safety comply with requirements. M3 carry out return of non-compliant raw materials and packaging to the suppliers. Corrective actions (M3) are also necessary when meat with pale, soft and exudative (PSE) and DFD defects is supplied. Additional information obtained in the study will support identification of previously non-normalized risks, will detail and justify the need for their regulation, and will aid in the development of effective measures to manage these risks with the provision of reasonable (non-regulated) investments.

Block 3, including the preparation of raw materials and consumer packaging, can be detailed processes as follows: defrosting meat raw materials; additional processing of carcasses and half-carcasses; cutting, boning, venation and sorting of meat; inspection, cleaning and washing of plant



ingredients; grinding; mixing. The processes are implemented in accordance with the objectives, norms (C1- Cn) and research results. Depending on the results of quality assurance/quality control and measurements (M4) of temperature and humidity parameters, organoleptic characteristics of the mixture after grinding and/or mixing, and the results of microbiological studies, corrective solutions (M3) develop to change the technological parameters of the system.

Block 4 - packaging of ingredients or a mixture of ingredients in consumer packaging and its sealing are important safety features of the finished canned meat pieces. Compliance with microbiological and temperature-time parameters will identify the residual risk with the existing approach of controls and management methods. Depending on the results, corrective solutions (M3) will be developed to change the technological parameters of the subsequent block or adjust the strategy of the process as a whole. For example, the total microbiological contamination level of canned food before sterilization should not exceed the normalized level provided in documentation. However, it will increase if the temperature parameters are violated and the product is delayed between the capping and the beginning of the sterilization process, which will lead to the production of unsafe products.

Block 5 – sterilization – is the determinative, formative indicator of the quality and safety of canned meat pieces. Exposure to high temperatures and pressures over a period of time determines the final quality and safety of the product, which must be maintained throughout its shelf life. Process parameters are normalized in proper, appropriate technological documentation (C1- Cn) depending on the raw material used, the type of consumer packaging and the achieved effect of the process lethality. Fluctuations of sterilization modes are possible for two reasons: the use of insufficiently accurately calculated sterilization mode, as well as inaccuracies and errors in the sterilization process, which entails the production of substandard products. The sterilization mode may not be sufficient for the manufacture of canned food that meets the requirements of industrial sterility, if the calculation does not take into account the uneven temperature field of the autoclave or the difference in the rate of heating of individual layers of liquid and particles in the flow-type heat exchangers. To eliminate this risk, a corrective solution (M3) is possible, i.e., increasing the duration of the sterilization regime by 10-20%.

#### 4. Conclusions

Control over product safety is declared to be the most important function of the state. In world practice, the principles of the HACCP system and the applied stages developed by the Codex Alimentarius Commission are focused on product safety risks [20]. The risks of spores of various microorganisms and their toxins occurring in canned meat pieces can be significant. Understanding the objectives of the assessment is a key factor in taking into account all types of technological and microbiological risks relevant to complex meat systems. Risk criteria include the determination of the acceptability or tolerability of the risk and the determination of its consequences. Important factors to be taken into account in such analysis are the duration of the risk assessment and the result of the risk occurring, as any increase in the duration of risk assessment increases the likelihood of a dangerous event before the risk can be assessed.

Work on the application of the methodology of systemic risk analysis in the production of canned meat in Russia has not yet been carried out. However, similar approaches are used, for example, in the assessment of production in the bakery industry [21], in the production of food ethanol [22], in the development of confectionery products [23].

The presented formal description of the technological process of canned meat in pieces allow lets us to consider the processing from the perspective of the universal methodology of systemic analysis. This allows us to: determine the general dependence of producing canned meat of suitably high quality and safety on informational, methodological support for the production process; track the risks and decision points; systematize the information, and; conduct deep, detailed analysis of the main components of the production blocks. This, in turn, serves as the basis for clear presentation and

proper analysis of knowledge so the technological processes can be correctly managed to improve the competitiveness, safety, environmental friendliness and efficiency of the meat canning industry.

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## ***Listeria monocytogenes* contamination in ready to eat foods**

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**Abstract.** *Listeria monocytogenes* is a food-borne pathogen responsible for listeriosis, a sickness with a high mortality rate. Listeriosis is largely associated with ready-to-eat (RTE) foods. It is well established that foods that pose the greatest risk of foodborne listeriosis are those RTE foods that have intrinsic characteristics such as pH and water activity that support the growth of *L. monocytogenes*. RTE foods can also become re-contaminated during further processing and handling. Increased handling leads to a higher probability of contamination. Sources of contamination can be food contact surfaces, processing machinery and workers. In our research, *L. monocytogenes* was detected in a RTE salad. Food safety criteria for *Listeria monocytogenes* in RTE foods have been applied from 2006 (Commission Regulation (EC) 2073/2005). Still, human invasive listeriosis was reported to increase during 2009-2013 in the European Union and European Economic Area. Time series analysis for the 2008-2015 period in this area showed an increasing trend of the monthly notified incidence rate of confirmed human invasive listeriosis of the over 75 age groups and female age group between 25 and 44 years old (probably related to pregnancies).

### **1. Introduction**

The genus *Listeria* currently includes 17 recognized species (*Listeria monocytogenes*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria welshimeri*, *Listeria marthii*, *Listeria innocua*, *Listeria grayi*, *Listeria fleischmannii*, *Listeria floridensis*, *Listeria aquatica*, *Listeria newyorkensis*, *Listeria cornellensis*, *Listeria rocourtiae*, *Listeria weihenstephanensis*, *Listeria grandensis*, *Listeria riparia*, and *Listeria booriae*) of small rod-shaped gram-positive bacteria. Only two of these species, *L. monocytogenes* and *L. ivanovii*, are considered pathogens. *L. monocytogenes* is an important human foodborne pathogen. Importantly, detection of *Listeria* species is often used by the food industry as a marker to detect conditions that allow for presence, growth, and persistence of *L. monocytogenes* [1].

The growth and survival of *L. monocytogenes* is influenced by a variety of factors. In food these include temperature, pH, water activity, salt and the presence of preservatives. The temperature range for growth of *L. monocytogenes* is between -1.5 and 45°C, with the optimal temperature being 30-37°C. Temperatures above 50°C are lethal to *L. monocytogenes*. As *L. monocytogenes* can grow at temperatures as low as 0°C, it has the potential to grow, albeit slowly, in food during refrigerated storage. *L. monocytogenes* will grow in a broad pH range of 4.0-9.6. Although growth at pH<4.0 has



not been documented, *L. monocytogenes* appears to be relatively tolerant to acidic conditions. *L. monocytogenes* becomes more sensitive to acidic conditions at higher temperatures [2]. *L. monocytogenes* is reasonably tolerant to salt and has been reported to grow in 13-14% sodium chloride [3]. Survival in the presence of salt is influenced by the storage temperature. Studies have indicated that in concentrated salt solutions, the survival rate of *L. monocytogenes* is higher when the temperature is lower [2].

Food safety criteria for *Listeria monocytogenes* in ready-to-eat (RTE) foods have been applied from 2006 (Commission Regulation (EC) 2073/2005). This Regulation came into force in January 2006 and requires the following:

- In RTE products intended for infants and for special medical purposes, *L. monocytogenes* must not be present in 25 g of sample (10 sample units);
- *L. monocytogenes* must not be present in levels exceeding 100 colony forming units per gram (CFU/g) during the shelf life of other RTE products (five sample units), and;
- In RTE foods that are able to support the growth of the bacterium, *L. monocytogenes* must not be present in 25 g of sample at the time of leaving the production plant (five sample units); however, if the producer can demonstrate, to the satisfaction of the Competent Authority (CA), that the product will not exceed the limit of 100 CFU/g throughout its shelf life, this criterion does not apply [4].

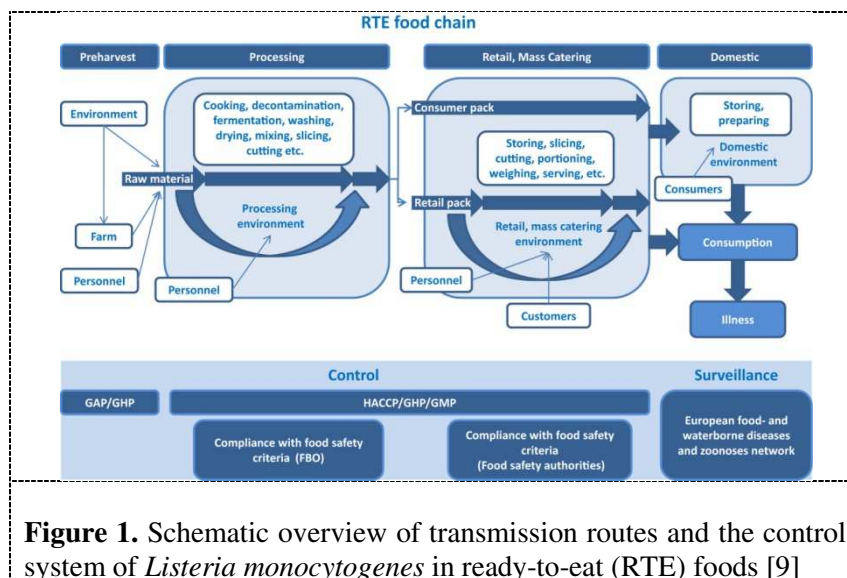
Still, human invasive listeriosis was reported to increase over the period 2009-2013 in the European Union and European Economic Area [5]. A conceptual model was used to identify factors in the food chain as potential drivers for *L. monocytogenes* contamination of RTE foods and listeriosis. Factors were related to the host (i. population size of elderly and/or susceptible people; ii. underlying condition rate), the food (iii. *L. monocytogenes* prevalence in RTE food at retail; iv. *L. monocytogenes* concentration in RTE food at retail; v. storage conditions after retail; vi. consumption), the national surveillance systems (vii. improved surveillance), and/or the bacterium (viii. virulence).

RTE food processing can involve, among other processes, comminution, addition of flavourings, binders, extenders and emulsifiers, etc., addition of preservatives (e.g. lactate, sodium nitrite), decontamination (water, acid), heating (e.g. pasteurising, cooking, baking, boiling, steaming), curing, smoking (hot or cold), fermentation and drying. Most of these steps have the potential to reduce pathogen loads on the RTE food at the time of consumption through microbial inactivation or inhibition of growth. The effectiveness of the control measures depends on the type of food and design of the process. In the case of a mild process (i.e. washing), the pathogen might survive while more intense or severe processes (i.e. sufficient heating) can lead to the elimination of the pathogen. RTE foods may also become re-contaminated during further processing and handling. In the latter case, increased handling leads to a higher probability of contamination [6]. Sources of contamination include food contact surfaces, processing machinery and workers. Contamination with *L. monocytogenes* after heat processing during further handling is one of the most important reasons for contamination. This is due to the capability of *L. monocytogenes* to form biofilms that result in enhanced resistance to disinfectants and antimicrobial agents.

RTE foods can be packed aerobically, under vacuum or in modified atmospheres. Packaging atmosphere can affect the growth of the pathogen during storage and, hence, the final risk. In addition, the amount of growth of *L. monocytogenes* can be affected by the assigned use-by date, since determines the storage time of the product.

Contamination of RTE food in packages that are opened and handled in retail stores (chubs, bricks, etc.) can also occur. Food retail and mass catering establishments are very different from food processing plants. They are open to the public, with customers, sales people, employees, and deliveries coming into the establishment. This can trigger the introduction of *L. monocytogenes* at various points and times of the day. *L. monocytogenes* is regularly found and often widely distributed in retail facilities [7-9]. Retail practices can result in cross-contamination from one RTE product to another, or in contamination from the retail environment, or in both [8]. The persistence of *L. monocytogenes* in a particular environmental site (i.e. slicing machine) at retail can be a niche that facilitates continued

cross-contamination of products from environmental sources. Surveys report that RTE delicatessen meats handled at retail stores have, in general, higher contamination than prepackaged products, indicating the possibility of cross-contamination at retail level [7,8,10] (Figure 1).



**Figure 1.** Schematic overview of transmission routes and the control system of *Listeria monocytogenes* in ready-to-eat (RTE) foods [9]

Some antimicrobial substances are added to food during production. For example, sorbic acid is sometimes added to prevent the growth of *L. monocytogenes* in foods such as cheeses, and a combination of sorbic acid and benzoic acid is commonly added to prevent the growth of *L. monocytogenes* in foods such as delicatessen-type salads.

Data reported in the zoonoses database show that from 2008 to 2015, as many as 525 human cases of listeriosis, 182 hospitalisations and 37 deaths were reported. Thus, most invasive listeriosis cases appear as sporadic infections and the detected outbreaks are usually small. The 'dairy' food category was responsible for four of these outbreaks causing 44 cases, while 'fish and seafood' and 'meat and meat products' food categories were responsible for 7 and 11 of these outbreaks causing 40 and 126 cases, respectively. In total, these three categories caused 22 (or 59%) strong-evidence food-borne outbreaks, 210 (or 40%) human cases, 125 (69%) hospitalisations and 26 (or 70%) deaths. Food of non-animal origin caused two outbreaks and 34 cases. Some of the outbreaks where the 'other' food category was implicated as the food vehicle could include RTE foods from the three food categories focused on in this Scientific Opinion [9], e.g. sandwiches, buffet meal, mixed foods.

Listeriosis is a very dangerous zoonosis, which, despite its low incidence, remains a major public health concern due to high mortality rate. Several studies have shown that RTE food is one of the most important vehicles responsible for human infections. The aim of this study was to establish the occurrence of *Listeria* spp., especially *L. monocytogenes* in ready to eat RTE food in Serbia.

## 2. Materials and Methods

Isolation of *L. monocytogenes* was performed according to the standard method [11]

## 3. Results and discussion

This study was conducted for one year – from January 2018 to December 2018. Four hundred and eighty-nine samples were collected. *L. monocytogenes* was found in one sample of vegetable salad with mayonnaise.

As *L. monocytogenes* can be found in the environment of food processing plants, RTE food processors should have an effective GMP programme with HACCP system to minimize all potential

sources of food contamination. These should address *L. monocytogenes* in the processing environment. In this regard, the importance of sanitation should not be overlooked. Sanitation management can lead to intervention innovations (e.g., effective remediation) and sanitary design improvements (e.g., equipment and facility). RTE food processors should also strongly consider introducing within their food safety systems one or more validated controls for the elimination of *L. monocytogenes* from their products (e.g., use of a post-lethality treatment). Furthermore, environmental and end-product sampling schemes and the use of microbiological testing as a verification tool to demonstrate the efficacy of the control measures put in place to address *L. monocytogenes* are recommended. Food processing plants should carry out regular environmental sampling to verify the effectiveness of their sanitation program for controlling *Listeria* in the plant environment, and should increase sanitation efforts and control measures in areas where *Listeria* are found.

#### 4. Conclusion

The public health risk from *L. monocytogenes* in RTE food depends on the effectiveness of the control and monitoring procedures which include good agricultural practice at the farm stage and the hazard analysis and critical control points (HACCP) programme and good hygiene practices (GHP) at the processing and retail stages as well as sampling procedures to evaluate compliance with the FSC for *L. monocytogenes*. In this study, the low prevalence of *L. monocytogenes* in RTE foods indicates this pathogen is a low risk for public health.

#### Acknowledgment

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## Influence of two different culture media on biofilm formation by *Listeria monocytogenes* isolated from a small-scale meat processing facility

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**Abstract.** In this study, 20 *Listeria monocytogenes* isolates detected in a food processing environment and food products were tested for biofilm-forming ability in two different culture media: Tryptone Soya Broth and Luria Bertani Broth. Statistical analysis of the data obtained was performed with the MINITAB software package, version 16.0. The two-sample t-test and confidence interval were used for data analysis. Significant differences between the isolates were observed in the ability to form biofilms.

### 1. Introduction

A foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis, a severe disease with high hospitalisation and case fatality rates. It can survive and grow in a wide range of adverse environmental conditions typical of food processing and preservation [1]. A critical point is the risk of *L. monocytogenes* persistence in food industry equipment and produce due to its ability to form biofilms [2]. Having colonised the food processing environment, *L. monocytogenes* can spread throughout the facility via aerosols, personnel, food workflows and contaminated contact materials leading to its persistent presence if sanitation procedures are insufficient [3]. In recent years, several authors investigated the relationships between biofilms and the main factors, such as surface type, temperature, and the presence of growth media and other microbes, involved in their formation, but the conclusions were often divergent [4,5,6]. Therefore, the aim of this study was to investigate the influence of two different culture media on biofilm formation by *L. monocytogenes* isolates from a small-scale meat processing facility. Also, the susceptibility of the isolates to several antibiotics was assessed.





## 2. Materials and methods

### 2.1. Origin of isolates

Twenty *L. monocytogenes* isolates originating from traditional meat products and environmental swabs were studied. These samples were taken during the slaughter and preparation of dry and smoked meat products in a four-year period. The collected samples were transported to the laboratory within 2 h in a cold bin at  $< 4^{\circ}\text{C}$ .

### 2.2. Culture media

The culture media used in this study were Tryptone soya broth (TSB) (CM0129, Oxoid, Basingstoke, UK) and Luria Bertani (LB) broth, (Becton, Dickinson and Company, Sparks, USA).

### 2.3. Microplate biofilm assay

The *L. monocytogenes* isolates were examined for their ability to form biofilms using the microplate assay [7]. Each isolate was inoculated in 3 mL TSB and multiplied at  $37^{\circ}\text{C}$  for 18 h. On the following day, 20  $\mu\text{L}$  of each isolate suspension were inoculated into four wells of sterile flat-bottom microtitre plates (Nunc, Roskilde, Denmark) and 150  $\mu\text{L}$  aliquots of the corresponding medium (TSB or LB broth) were added into each well. The plates also included a set of eight wells filled only with the tested medium as a negative control. After incubation at  $30^{\circ}\text{C}$  for 72 h, the plates were washed three times with sterile saline and allowed to dry at room temperature. The attached bacteria were fixed for 20 minutes at room temperature by adding 200  $\mu\text{L}$  volumes of methanol into each well.

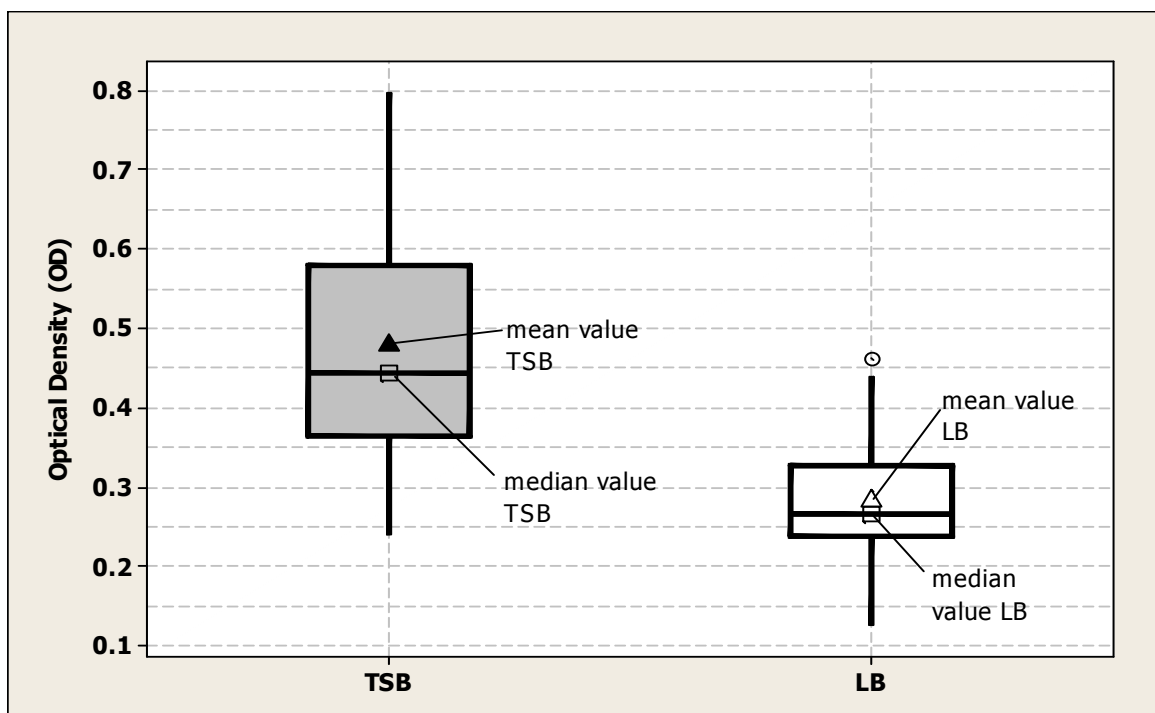
The plates were stained with 200  $\mu\text{L}$  0.3% aqueous solution of crystal violet (Crystal Violet, Fluka) for 30 minutes at room temperature. After being stained, the plates were rinsed under running water until there was no visible trace of stain. The stain bound to bacteria was dissolved by adding 200  $\mu\text{L}$  of 96% ethanol. Optical density (OD) was measured spectrophotometrically (Labsystems Multiscan® MCC/340) using a 595 nm filter. Cut-off OD (OD<sub>c</sub>) was defined as three standard deviations above the mean OD of the negative control.

### 2.4. Antibiotic susceptibility testing

The antimicrobial susceptibility of *L. monocytogenes* isolates to antibiotics was assessed using the standard disc diffusion test on Mueller Hinton agar (CM0337, Oxoid, UK), in line with the guidelines of the Clinical and Laboratory Standards Institute [9]. The following antibiotics were used: penicillin (P, 10 U), amoxicillin/clavulanic acid (AMC, 20/10  $\mu\text{g}$ ), ampicillin (AMP, 10  $\mu\text{g}$ ), ceftriaxone (CRO, 30  $\mu\text{g}$ ), cefotaxime (CTX, 30  $\mu\text{g}$ ), ciprofloxacin (CIP, 5  $\mu\text{g}$ ), erythromycin (ERY, 15  $\mu\text{g}$ ), chloramphenicol (CHL, 30  $\mu\text{g}$ ), nalidixic acid (NA, 30  $\mu\text{g}$ ) and trimethoprim/sulfamethoxazole (SXT, 1.25/23.75  $\mu\text{g}$ ).

## 3. Results and discussion

Our results demonstrated a strong influence of nutrient availability on biofilm production by *L. monocytogenes*. Statistical analysis was performed by the MINITAB software package, version 16.0. Concentrations were expressed as mean values, standard deviations, median and range of minimum to maximum. The two-sample t-test and confidence interval were used to examine statistical differences of transformed data between two groups of samples analysed. Significant differences between two groups of isolates were observed ( $p = 0.000$ ;  $p < 0.05$ ) in their ability to form biofilms. Box plots were used to illustrate both the measures of central tendency and the variability of the data on the distribution of biofilm formation by *L. monocytogenes* isolates grown in TSB and LB medium (Figure 1).



**Figure 1.** Box plot diagram of biofilm formation by 20 *L. monocytogenes* isolates grown in TSB and LB medium. Bacteria were incubated at 30 °C for 72 h. The differences were considered significant if  $p < 0.05$ .

The ability of *L. monocytogenes* to survive in extreme conditions and to form biofilms on various surfaces is a significant challenge for food safety [9]. Biofilm formation creates major problems in the food industry because it can be an important source of food contamination [9].

All *L. monocytogenes* isolates proved susceptible to beta-lactam antibiotics (penicillin, ampicillin and amoxicillin/clavulanic acid), macrolides (erythromycin), sulphonamide (trimethoprim/sulfamethoxazole), chloramphenicol and resistant to nalidixic acid. Cefotaxime resistance was detected in 7 *L. monocytogenes* isolates obtained from minced meat samples taken from a machine, or from a mixer, dry sausage (pork), dry pancetta-sliced, pork tenderloin, pancetta or pork neck. These findings were similar to the study conducted by [10], who reported the majority of *Listeria* spp. isolated from food, clinical and environmental samples are sensitive to ordinarily used antibiotic therapy that is usually applied against Gram-positive bacteria including tetracyclines, ampicillin, penicillin G, imipenem, amoxicillin, sulphonamides, aminoglycosides, macrolides, chloramphenicol and glycopeptides.

#### 4. Conclusion

Results of the microtitre plate based crystal violet assay revealed that all *L. monocytogenes* isolates produced biofilm on polystyrene. The nutrient-rich medium, i.e. cultivation in TSB, significantly enhanced biofilm production. Plastic materials are now more widely used in the food industry for the construction of tanks, pipework, accessories and cutting surfaces where nutrients are commonly available. Therefore, biofilms of *L. monocytogenes* are considered as a key factor contributing to the persistence of certain strains and repeated food contamination; the removal of irreversibly adhered cells is difficult and requires the application of strong mechanical force or chemical interruption of the adhesion using surfactants, sanifiers or heat. All *L. monocytogenes* isolates proved susceptible to beta lactam antibiotics, macrolides and sulphonamide, which are the first choice of antibiotics in the

therapy of listeriosis. Also, the resistance to the first generation fluoroquinolone (nalidixic acid) is considered as an intrinsic feature of this type of bacteria.

### Acknowledgment

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## Sodium and potassium contents and ratios in pork stews produced with lower amounts of sodium chloride

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**Abstract.** The goal of this study was to investigate the influence on the sodium:potassium ratio of reducing sodium chloride content in pork stew by partial replacement with other two chloride salts. The trial consisted of five groups. In the control pork stew, only sodium chloride was added. In group 1, one third of the sodium chloride was replaced with potassium chloride; in group 2, one half of the sodium chloride was replaced with potassium chloride; in group 3, sodium chloride was reduced by half and one quarter of ammonium chloride (in relation to the control group) was added, and in group 4, sodium chloride was reduced by 62.5% and an equal amount of ammonium chloride was added. Moderate reduction of sodium content was achieved in group 1 (46.19%), while greater reductions of sodium content were achieved in groups 2, 3 and 4 (these had 64.69%, 61.54% and 67.91% less sodium than did the control, respectively). Potassium content increases were determined in groups 1 and 2 (potassium levels were 38.71% and 50.27% greater than in the control, respectively). The best sodium:potassium ratio was achieved in group 2 pork stew (0.47), in which half the sodium chloride was replaced with potassium chloride.

### 1. Introduction

Reducing sodium/sodium chloride in food, particularly in meat products, has become one of the main dietary aims nowadays, and will also be important in the future. Daily sodium intake often exceeds the level, as advised by the World Health Organization, that can cause several debilitating health effects, primarily linked to essential hypertension and consequent cardiovascular disorders. It has been estimated that 62% of stroke and 49% of coronary heart disease is caused by high blood pressure [1]. Sodium chloride is commonly used in all meat products and it has an important role as the prototypical stimulus for salty taste [2]. Saltiness perception is a very complex system and can be explained by the unique sodium-specific transduction mechanism involving epithelial sodium channels (ENaCs) on the taste receptor cells [3]. Beside saltiness, sodium chloride improves the sensory properties of food by decreasing bitterness and increasing sweetness [4]. Due to these desirable effects of sodium chloride, it is a great challenge to reduce its content in food. Use of other salts as partial replacers of sodium chloride could be a way to reduce the sodium content of meat products, but this is also linked to the



appearance of bitter and metallic tastes. The most common replacer is potassium chloride, followed by magnesium and calcium salts and ascorbates [5]. Ammonium chloride can also be used in combination with autolysed yeast [6]. Commercial use of sodium chloride replacers is still restricted, but presently, potassium chloride and ammonium chloride are both recognized as safe.

The topic of reducing sodium content in meat products pertains to many investigations on cooked sausages, fermented sausages and dry meat products, but there are few literature data about sodium reduction in prepared meat meals. Prepared and ready-to-eat meals have become an important choice for modern people with respect to their fast lifestyles, particularly in developed countries. Due to that, the goal of this study was to investigate the impact of reducing the sodium chloride content in pork stew by partial replacement of sodium chloride with potassium chloride or ammonium chloride with a target to achieve a better sodium to potassium ratio.

## 2. Materials and methods

### 2.1. Trial design

The trial consisted of five groups of pork stew with the compositions presented in Table 1. In the control, only sodium chloride was added. In group 1, one third of the control amount of sodium chloride was replaced with potassium chloride, while in group 2, one half of the sodium chloride was replaced with potassium chloride. In group 3, the control amount of sodium chloride was reduced by one half and one quarter of ammonium chloride (in relation to the sodium chloride in the control) was added. In group 4, sodium chloride was reduced by 62.5% of the amount added to the control and an equal amount of ammonium chloride (as sodium chloride) was added.

### 2.2. Meal preparation

Minced onion was fried in the sunflower oil for 20 minutes and after that, red pepper, salts and meat (pork shoulder) cut into pieces were added, as well as water. Stews were cooked for 80 minutes. After cooking, samples of stew were put into plastic containers and refrigerated until analysis.

**Table 1.** Stew composition

Group	Onion, g	Sunflower oil, mL	Meat, g	Red pepper g	Water, mL	Sodium chloride, g	Potassium chloride, g	Ammonium chloride, g
Control	500	50	500	5.00	500	16.00	-	-
1	500	50	500	5.00	500	10.66	5.33	-
2	500	50	500	5.00	500	8.00	8.00	-
3	500	50	500	5.00	500	8.00	-	4.00
4	500	50	500	5.00	500	6.00	-	6.00

### 2.3. Determination of Na and K

To digest the stews, approximately 0.3 g amounts were transferred into Teflon vessels and 5 mL nitric acid (p.a. Sigma-Aldrich, St. Louis, MA, USA) and 1.5mL hydrogen peroxide (30%, p.a., Merck & Co., Whitehouse Station, New Jersey, USA.) were added. The sample solutions were quantitatively transferred into disposable flasks and diluted, then digested using a microwave program that consisted of three steps as follows: 5 min from room temperature to 180°C, 10 min hold 180°C, 20 min vent. After cooling at room temperature, digests were diluted to 100 mL with deionized water (ELGA). Analysis was performed by inductively-coupled plasma mass spectrometry (ICP-MS). Measurements were obtained using the instrument iCap Q (Thermo Scientific, Bremen, Germany), equipped with

collision cell and operating in kinetic energy discrimination (KED) mode. The following isotopes were measured:  $^{39}\text{K}$  and  $^{23}\text{Na}$ .

Torch position, ion optics and detector settings were adjusted daily using tuning solution (Thermo Scientific Tune B) in order to optimize measurements and minimize possible interferences. For the qualitative analysis of the samples, a five-point calibration curve (including zero) was constructed for each isotope in the concentration range of 0.1-2.0 mg/L. An additional line of the peristaltic pump was used for on-line introduction of multi-element internal standard ( $^6\text{Li}$ ,  $^{45}\text{Sc}$  – 10 ng/mL;  $^{71}\text{Ga}$ ,  $^{89}\text{Y}$ ,  $^{209}\text{Bi}$  – 2 ng/mL) covering a wide mass range. Concentrations of each measured isotope were corrected for response factors of both higher and lower mass internal standard using the interpolation method. The quality of the analytical process was controlled by the analysis of the standard reference material (NIST SRM 1577c). Measured concentrations were within the range of the certified values for all isotopes.

### 3. Results and discussion

All experimental samples of pork stew were sensorially acceptable [7].

Results of sodium and potassium content in pork stew are presented in Table 2. Sodium content was directly influenced by the amount of added sodium chloride, and the highest sodium content was determined in the control pork stew. Moderate reduction of sodium content was achieved in group 1 (46.19% less sodium than the control), in which the amount of sodium chloride was one third less than in the control. Greater reductions of sodium content were achieved in groups 2, 3 and 4 (64.69%, 61.54% and 67.91% less sodium than the control, respectively). In these stews, the amount of sodium chloride was half that of the control (groups 2 and 3), while in group 4, the sodium chloride content was 62.5% of that of the control.

Potassium content was directly influenced by adding potassium chloride to the experimental pork stews. Potassium content increased in groups 1 and 2 (38.71% and 50.27% more potassium than the control, respectively). In groups 3 and 4, potassium contents were lower than in the control, by 21.86% and 17.97%, respectively, due to potassium chloride not being added to these products.

The highest sodium:potassium ratio was in the control pork stew (1.99). However, the best sodium:potassium ratio was achieved in group 2 pork stew (0.47), in which half the sodium chloride was replaced with potassium chloride, bringing the ratio into accord with level of 0.43 that is recommended by the World Health Organization [8]. The sodium:potassium ratios in groups 1 and 4 were similar (0.77 and 0.78, respectively), while that of group 3 was 0.98.

**Table 2.** Sodium and potassium content in pork stew, mg/kg

Group	Sodium	Potassium	Sodium:Potassium ratio
Control	11591.27	5838.61	1.99
1	6237.78	8099.01	0.77
2	4092.86	8773.81	0.47
3	4458.14	4562.02	0.98
4	3719.92	4789.25	0.78

### 4. Conclusion

The highest sodium content was determined in the control pork stew, due to this having the highest amount of added sodium chloride.

Moderate sodium reduction was achieved in experimental pork stews in which sodium chloride was partially replaced with potassium chloride or ammonium chloride and in which, at the same time, the amount of added sodium chloride was reduced.

The highest potassium contents were determined in the groups 1 and 2 pork stews, produced with added potassium chloride.

In all stews from experimental groups, the sodium:potassium ratio was better than in the control pork stew, and without particular impact on sensory attributes of the products.

### Acknowledgement

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## Chemical composition and fatty acid profile of the *longissimus dorsi* muscle in Simmental bulls

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**Abstract.** The aim of this paper was to evaluate the carcass traits (live weight at slaughter, hot and cold carcass weights, dressing percentage, chilling loss), chemical composition and fatty acid profile of *longissimus dorsi* muscle (MLD) of Simmental bulls. The investigation was carried out on 10 Simmental bulls fattened in intensive conditions. The live, hot and cold carcass weights at slaughter were 597.9±29.53 kg, 326.9±17.06 kg and 319.4±16.64 kg, respectively. Dressing percentage was 54.6±1.17% and chilling loss was 2.3±0.26%. The mean muscle chemical composition was: dry matter 24.14 ±0.19%, water 75.86 ±0.59%, protein 20.78 ±0.30%, intramuscular fat 2.35 ±0.39%, ash 1.01±0.05%. High correlation was detected between live weight, hot carcass weight and cold carcass weight. The fatty acids of intramuscular fat consisted of 48.02±0.99%, 46.47±1.30% and 5.51±0.28% saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), respectively. The PUFA/SFA ratio was low (around 0.1), but the n-6/n-3 PUFA ratio was 4.24 and close to the recommended value. The level of MUFA correlated highly with the  $\Delta 9$ -desaturase (18) index ( $r=0.82$   $p<0.05$ ) and oleic acid (C18:1n-9) ( $r=0.90$   $p<0.05$ ), as did PUFA and linoleic acid (C18:2n-6) ( $r=0.96$   $p<0.05$ ). Correlations ( $p<0.05$ ), between major fatty acids, live weight and intramuscular fat were weak.

### 1. Introduction

Over recent decades, growing attention has been paid to dietary aspects of bovine meat consumption. Health concerns have been directed at the fat content and fatty acid composition of beef, particularly due to the high content of saturated fatty acids (SFA) which are believed to be associated with some human diseases [1]. The fatty acid composition of beef is influenced by a number of factors, including diet, breed, genotype, age and gender. Genetic and nutritional approaches have been widely studied in relation to fatty acid composition of beef, although it is acknowledged that genetic factors generally provide smaller differences than animal dietary factors. Increasing the content of n-3 polyunsaturated fatty acids (PUFA) and reducing SFA with the net effect of increasing the PUFA/SFA ratio and reducing the n-6/n-3 PUFA ratio in beef intramuscular fat are important priorities [2].

Simmental cattle are the most common dual-purpose breed in Serbia that accounts for up to 75% of the overall cattle population [3]. A common diet used for beef cattle production under indoor conditions consists of maize silage and a concentrate including soybean. The objective of this paper was to analyse the carcass characteristics (live weight at slaughter, hot and cold carcass weight,





dressing percentage, chilling loss), the chemical composition and fatty acid profile of the *longissimus dorsi* muscle in young Simmental bulls and compare them with results from the literature for the same and different breeds of cattle.

## 2. Materials and Methods

### 2.1. Sample collection

The investigation was carried out on 10 Simmental bulls, fattened in intensive conditions, approximately 16 months old. We analysed carcass characteristic (live weight at slaughter, hot and cold carcass weight, dressing percentage, chilling loss), chemical composition and fatty acid profile of *longissimus dorsi* muscle (MLD). Cattle were slaughtered at a commercial slaughterhouse. Live weight at slaughter (LWS) and hot carcass weight (HCW) were recorded on the slaughter day. After a chilling period of approximately 24 hours at 4°C, cold carcass weight (CCW) was measured and samples of the *longissimus dorsi* muscle were collected at the 9th-10th thoracic vertebrae. Dressing percentage was calculated as the ratio of HCW to LWS. Chilling loss was calculated as the ratio of HCW to CCW.

### 2.2. Chemical and fatty acid analysis

Moisture content was calibrated by the standard oven drying method [4], fat content by Soxhlet extraction [5], protein content according to ISO [6] and ash content according to ISO [7].

FA analysis was by capillary gas chromatography. In brief, the total lipids were extracted from the meat by accelerated solvent extraction (ASE), (ASE 200, Dionex, Sunnyvale, CA, USA) with petroleum ether and isopropanol mixture (60:40, v/v) (as proposed by Dionex Application Note No. 345) at 100°C over three static cycles under nitrogen at 12 MPa. The solvent from the collected extracts was removed under a stream of nitrogen (Dionex Solvent evaporator 500) at 50°C until dry. Fatty acid methyl esters (FAMES) in the extracted lipids were prepared by esterification using 0.5 M sodium methoxide in anhydrous methanol as proposed by Christie *et al.* [8]. FAMES were determined by gas-liquid chromatography (Shimadzu 2010, Kyoto, Japan) equipped with a flame ionization detector and capillary HP-88 column (length 100m, i.d. 0.25 mm, film thickness 0.20 µm). Injector and detector temperature were 250°C and 280°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 1.87 mL min<sup>-1</sup>. The injector split ratio was set at 1:50. A programmed column oven temperature starting at 50°C and ending at 230°C was applied. Total analysis time was 66.5 min. The chromatographic peaks in the samples were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, PA) and a standard mixture of methyl esters of cis-9,11 and trans-10,12 isomers of conjugated linoleic acid (CLA) (O5632 ≥99%, Sigma-Aldrich, USA). Each sample was analysed in duplicate. Results were expressed as a percentage by weight of the total identified fatty acids. Indexes of Δ9-desaturase enzyme activity were calculated using formulas described by Bureš *et al.* [9].

### 2.3. Statistical analysis

Statistical analyses of the results were conducted using software GraphPad Prism version 7.0 for Windows (GraphPad Software, San Diego California USA, [www.graph.com](http://www.graph.com)). All parameters were described by descriptive statistics (mean, standard deviation). Pearson's correlation was used to determine relationships between means of the examined parameters. Statistical differences between means of the examined parameters were determined on the level  $p < 0.05$ .

## 3. Results and discussion

The present study was conducted to evaluate the carcass traits, chemical and fatty acid composition of the *longissimus dorsi* muscle of Simmental bulls, fattened in intensive conditions. The live weight at slaughter, carcass traits (hot and cold weight, dressing percentage chilling loss) and the chemical composition of the *longissimus dorsi* muscle are presented in Table 1. The mean live weight recorded

was  $597.9 \pm 29.53$  kg, which is slightly lower than found for Czech Fleckvieh cattle, fattened to approximately same age [10]. However, our young bulls had a higher average final mass compared to the mass recorded by Štoković *et al.* [11] for Croatian Simmental cattle.

**Table 1.** Mean live weight, carcass quality traits and chemical composition of the *longissimus dorsi* muscle in 10 Simmental cattle

Trait	$\bar{x}$	SD
Live weight at slaughter (kg)	597.9	29.53
Hot carcass weight (kg)	326.9	17.06
Cold carcass weight (kg)	319.4	16.64
Dressing percentage (%)	54.6	1.17
Chilling loss (%)	2.3	0.26
<b><i>Longissimus dorsi</i> composition</b>		
Dry matter (%)	24.14	0.19
Moister (%)	75.86	0.59
Intramuscular fat (%)	2.35	0.39
Protein (%)	20.78	0.30
Ash (%)	1.01	0.05

$\bar{x}$ : mean; SD: standard deviation

High correlation was detected between LWS, HCW and CCW, as expected. However, LWS had no influence on the chemical composition of the MLD. The dressing percentage was  $54.6 \pm 1.17\%$  and this result accorded with those in other studies [12,13,14].

The chemical analysis of the *longissimus dorsi* muscle showed lower intramuscular fat content,  $2.35 \pm 0.39\%$ , than in literature records [15,11]. According to our findings, this Simmental beef could be classified as lean meat, because intramuscular fat content of 2-5% in many countries is accepted as being low in fat [2].

The results of the evaluations fatty acid composition showed that the total fatty acid content of the intramuscular fat consisted, on average, of  $48.02 \pm 0.99\%$  SFA,  $46.47 \pm 1.30\%$  MUFA and  $5.51 \pm 0.28\%$  PUFA (Table 2).

The predominant SFAs were myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0). Palmitic acid (C16:0) made up the greatest proportion of SFA, which is in agreement to the literature records [2,16]. The medium-chain SFAs, lauric (C12:0), myristic (C14:0) and palmitic (C16:0) acid, are nutritionally undesirable because they adversely affect plasma cholesterol level. They increase the level of blood low-density lipoproteins, which are thought to be associated with an increased risk of coronary heart disease [17]. Among the monounsaturated fatty acids detected in the intramuscular fat in the current study, and those with the highest percentages were oleic (C18:1cis-9) and palmitoleic (C16:1n-7) fatty acids ( $42.89 \pm 1.16\%$  and  $3.58 \pm 0.10\%$ , respectively). Oleic (C18:1cis-9) and stearic (C18:0) fatty acids constituted more than 50% of the total fatty acid in all reported studies. MUFAs of the cis-configuration are hypocholesterolemic with the added advantage of not reducing high-density lipoprotein that protects against coronary heart disease (CHD) [18]. Stearoyl-CoA desaturase ( $\Delta 9$ -desaturase) is the enzyme responsible for conversion of SFA into MUFA in mammalian adipocytes. In case of ruminants, fatty acids in the feed are chemically reduced by microorganisms in the rumen and are adsorbed as SFAs. The composition of fatty acids stored in the fat depots reflects the previous action of  $\Delta 9$ -desaturase on substrates such as C18:0 or C16:0. In our experiment, the scores for  $\Delta 9$ -desaturase (16) index and  $\Delta 9$ -desaturase (18) index ( $11.87 \pm 0.32$  and  $72.16 \pm 0.77$  respectively), were similar to values found in literature for Simmental cattle [10,9]. As expected, the level of MUFA

correlated highly with  $\Delta 9$ -desaturase (18) index ( $r=0.82$   $p<0.05$ ) and oleic acid (C18:1n-9) ( $r=0.90$   $p<0.05$ ).

**Table 2.** Fatty acid profile (% of total fatty acids), fatty acid ratios and desaturase indexes of the *longissimus dorsi* muscle from 10 Simmental cattle

Fatty acid	$\bar{x}$	SD
C14:0	1.70	0.63
C15:0	0.48	0.13
C16:0	26.56	0.04
C17:0	0.71	0.02
C18:0	16.54	0.15
C20:0	0.09	0.02
C16:1n-7	3.58	0.10
C18:1n-9	42.89	1.16
C18:2n-6	4.18	0.12
C18:3n-3	0.96	0.04
C20:4n-6	0.28	0.03
C22:5n-3	0.06	0.08
C22:6n-3	0.03	0.01
SFA <sup>a</sup>	48.02	0.99
MUFA <sup>b</sup>	46.47	1.30
PUFA <sup>c</sup>	5.51	0.28
PUFA/SFA ratio	0.11	0.02
n-6/n-3 ratio <sup>d</sup>	4.24	0.72
$\Delta 9$ -desaturase (16) index <sup>e</sup>	11.87	0.32
$\Delta 9$ -desaturase (18) index <sup>f</sup>	72.16	0.77

$\bar{x}$ : mean; SD: standard deviation; <sup>a</sup>SFA: saturated fatty acids = C14:0 + C15:0 + C16:0 + C17:0 + C18:0 + C20:0; <sup>b</sup>MUFA: monounsaturated fatty acids = C16:1 + C18:1n-9; <sup>c</sup>PUFA: polyunsaturated fatty acids = C18:2n-6 + C18:3n-3 + C20:4n-6 + C20:5n-3 + C22:5n-3; <sup>d</sup>n-6/n-3 ratio = (C18:2n-6 + C20:4n-6) / (C18:3n-3 + C20:5n-3 + C22:5n-3); <sup>e</sup> $\Delta 9$ -desaturase (16) index =  $100 * [C16:1n-7 / (C16:0 + C16:1n-7)]$ ; <sup>f</sup> $\Delta 9$ -desaturase (18) index =  $100 * [C18:1n-9 / (C18:0 + C18:1n-9)]$

Total PUFA in our Simmental muscle was notably lower than values reported for Simmental cattle [9,16], but slightly higher than found by [11] for Croatian Simmental cattle. A high correlation was detected between total PUFA and linoleic acid (C18:2n-6) ( $r=0.96$   $p<0.05$ ). In our study, the P/S ratio was 0.11, which is highly unfavourable from a human dietary aspect. The minimum P/S ratio advised for human nutrition is at least 0.45 [19] and generally should be around 0.7 [20]. Nevertheless, the P/S index is of limited significance as not all SFAs increase cholesterol, as has already been pointed out. Additionally, the positive effects of MUFAs like oleic acid are not considered when this index is used. From the human nutrition point of view, one of the most important indices widely used to evaluate the nutritional value of fat is the n-6/n-3 PUFA ratio, which should have a value of below 4 [21].

#### 4. Conclusion

The present study showed that the meat produced by young Simmental bulls is characterized by a low intramuscular fat content. Sex, age and weight are probably the main reason for this low fat content in the muscle studied. The fat content in muscle has an effect on the fatty acid composition, independent of animal dietary factors. Generally, it can be concluded that the relatively low proportion of intramuscular fat did lead to a suitably high proportion of PUFA and favourable balance n-6/n-3 PUFA, which was close to the recommended value in our study.

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# The influence of air hygiene on microbiological safety of butter

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**Abstract.** In this work, the microbial air quality in a butter factory was studied, and the microbial safety of packaged butter during its shelf-life is presented. Control of air hygiene was performed in two halls in one butter factory. The production hall housed the butter churner, and here, 20 kg blocks of butter were packaged in cardboard boxes (8 different locations were examined). The packaging hall housed a line for splitting the 20 kg blocks of butter into 125 g amounts and a butter packaging machine producing smaller cardboard boxes containing the 125 g packets of butter (6 locations were examined). The hygienic quality of air in the two halls was assessed by determining the numbers of mesophilic aerobic bacteria, yeasts and molds. Microbiological examination of bulk 20 kg butter blocks and 125 g packets of butter was conducted every tenth day over 60 days. The air in the production plant contained mesophilic bacteria that settled on plates during 20 minutes' exposure; numbers were from 6-20 at churning, and 12-36 colonies at packing. The mycological profile of packaged butter is largely a result of the presence of molds in the packaging hall air and their direct incorporation onto the finished product.

## 1. Introduction

The technological processing of food products in food processing plants leads to contamination of the air with phages, bacteria, yeasts and molds. Movement of air currents from ventilation systems can contribute to the spread of microbial aerosols in food plants. Therefore, great attention should be paid to air filtration in food plants. The passage of air through efficient filters retained up to 99.99% of microorganisms [1]. To this end, in food production plants, the use of filtration transmission devices creating laminar flow of sterile air in the vertical and horizontal planes is recommended [2].

During operations such as churning and packing, butter is exposed to the effects of contamination from the ambient air in the production plant and from the staff working in these areas, as well as to the impact of the ambient temperature. Therefore, in terms of maintaining good microbiological quality of the butter, control of the microbiological air quality in rooms where butter is churned and packaged is necessary [3].

Manipulations during butter production and packaging can lead to undesirable water and residual or dispersed product on the floor. Subsequent movement and activity of workers at the facility and/or the processes of washing and cleaning equipment can result in dispersion of microorganisms in the form of aerosols into the air. Activities that promote increases in aerosols often produce unacceptable levels of air contamination [4]. Hence, it is desirable to maintain dry conditions in the production area.

Considering the packaging process, special attention must be given to the choice of the packaging material. Cardboard, as a secondary material, can be a significant source of mold spores, especially if





it is recycled. To prevent contamination, cardboard packaging material should not be handled in the production area but in a separated area. Condensation or humidity, if present, will initiate the growth of mold spores. Some authors [2] state there is a connection between microbiological contamination of food products and aerial spread of microorganisms. For example, the level of contamination of food increases by 120% after 48 h exposure to air at 32 °C, compared with 24 h exposure to the same conditions. Contamination of butter increases with increasing humidity, with 80-95% of isolated microorganisms being Gram-positive airborne bacteria (cocci, bacilli). Air quality is especially important for butter manufactured in continuous-type mixers, which can incorporate ambient air into the product at levels up to 5% of product volume [5].

## 2. Materials and methods

Control of air hygiene was performed in two halls in one butter factory. The production hall housed the butter churning machine. Butter was accepted from the churning machine, and 20 kg blocks of butter were packaged by hand in polyethylene bags that were then placed in protective cardboard boxes (8 different locations were examined in this hall). After boxing, the butter blocks were stored at <6 °C. In the butter packaging hall, retail 125 g packs of butter were formed from the 20 kg blocks of butter and packaged in smaller cardboard boxes (6 locations).

Air sampling was carried out by exposure of open Petri dishes with the appropriate nutrient medium (nutrient agar for total viable count; TVC, and Sabouraud maltose agar with streptomycin (from 0.01 to 0.02%) for the total number of yeasts and molds). Petri dishes were exposed for 20 mins, after which the plates were incubated (TVC: 2-3 days, 30 °C; yeasts: 3-5 days, 25 °C; molds: 7 days, 25 °C), and colonies were counted.

Blocks of butter (20 kg) (60 samples) and 125g packs of butter (60 samples) were kept in refrigerated storage at 4 °C in order to monitor expiration dates. Microbiological control of these samples was conducted every ten days from the date of production, for a total of 60 days. The microbiological tests conducted were in accordance with: (a) TVC [6]; b) Lipolytic bacteria [7]; c) Proteolytic bacteria [7]; d) *Salmonella* spp. [8]; e) *Escherichia coli* [9]; f) Sulphate-reducing clostridia [10]; g) *Enterobacteriaceae* [11]; h) Coagulase positive staphylococci [12]; i) *Listeria* spp. and identification to species level using biochemical systems API List (BioMeriex, France) [13] and j) Yeasts and molds [14].

## 3. Results and discussion

TVC, yeasts, and molds expressed as colony-forming unit (cfu) determined in the air in the butter processing halls are shown in Table 1.

**Table 1.** Total viable counts, yeast and mold numbers determined in butter production and packaging halls (cfu deposited on settle plates during 20 min exposure to the air)

	TVC (cfu)	Yeast (cfu)	Molds (cfu)
<i>Production hall location</i>			
1	10	–	5
2	6	1	3
3	7	–	5
4	18	–	4
5	17	–	6
6	7	–	9
7	14	1	7
8	20	–	8

<i>Packaging hall location</i>			
1	28	1	8
2	15	–	10
3	12	–	12
4	36	1	7
5	21	–	15
6	19	–	9

The results in Table 1 show the TVC ranged from 6-20 colonies (production hall) to 12-36 colonies (packaging hall), suggesting poorer microbiological quality of air in the packaging hall. This suggests a potentially negative impact of the packaging hall on the microbiological safety of packaged butter, since all the manipulative packaging operations are conducted here.

A similar trend was evident in the total number of airborne molds, since air in the packaging hall contained more molds than air in the production hall. However, any airborne contamination in the production hall could have negative effects on the microbiological quality of bulk and packaged butter, due to the fact that up to 5% of the volume of churned butter can be ambient air with its accompanying load of microorganisms, incorporated into the butter at churning [5]. Similar values for TVC were found [15] in an examination of air hygiene in dairy plants in Belgrade. These authors stated the TVC in air in the locations examined ranged from 12 to 36 cfu, while the number of yeasts and molds was 2 to 7cfu [15].

Examination of air contaminated with molds in cheese ripening plants [16] resulted in identification of numerous molds that were classified in 11 genera and 32 species. The greatest number of species (45.16%) belonged to the genera *Penicillium* (mostly *Penicillium verrucosum* var. *cyclopium*) and *Aspergillus*. Some (38.71%) species of molds were indoor air contaminants in the cheese ripening plants studied [16]. Since 61.29% of molds appeared simultaneously as cheese contaminants during ripening and as airborne contaminants in these premises, the author concluded the air was the main source of contamination for these molds.

Results of microbiological testing of the two different butter pack types (butter blocks of 20 kg and butter packs of 125 g) during storage at 4 °C are shown in Tables 2 and 3, respectively.

**Table 2.** Results of microbiological examination (cfu/g or cfu/0.1 g or presence/absence) of 20 kg butter blocks during storage at 4 °C to determine shelf life

Microorganisms	Storage time (days)						
	1	10	20	30	40	50	60
TVC per g	–	–	–	–	–	2500	8000
Proteolytic bacteria per g	–	–	–	–	–	–	–
Lipolytic bacteria per g	–	–	–	–	–	9	98
<i>Salmonella</i> spp. presence/absence in 25 g	–	–	–	–	–	–	–
<i>Escherichia coli</i> per 0.1 g	–	–	–	–	–	–	–
Sulphate-reducing clostridia per 0.1 g	–	–	–	–	–	–	–
Coagulase positive staphylococci per 0.1 g	–	–	–	–	–	–	–
<i>Listeria</i> spp. presence/absence in 25 g	–	–	–	–	–	–	–
<i>Enterobacteriaceae</i> per g	–	–	–	–	–	2	6
Yeasts per g	–	–	–	–	–	–	–
Molds per g	–	–	–	–	3	11	22



**Table 3.** Results of microbiological examination (cfu/g or cfu/0.1 g or presence/absence) of 125 g butter packs during storage at 4 °C to determine shelf life

Microorganisms	Storage time (days)						
	1	10	20	30	40	50	60
TVC per g	–	–	–	2	80	4000	12000
Proteolytic bacteria per g	–	–	–	–	–	–	–
Lipolytic bacteria per g	–	–	–	2	11	28	73
<i>Salmonella</i> spp. presence/absence in 25 g	–	–	–	–	–	–	–
<i>Escherichia coli</i> per 0.1 g	–	–	–	–	–	–	–
Sulphate-reducing clostridia per 0.1 g	–	–	–	–	–	–	–
Coagulase positive staphylococci per 0.1 g	–	–	–	–	–	–	–
<i>Listeria</i> spp. presence/absence in 25 g	–	–	–	–	–	–	–
<i>Enterobacteriaceae</i> per g	–	–	–	1	1	8	12
Yeasts per g	–	–	–	–	–	1	2
Molds per g	–	–	–	4	15	52	89

The microbiological results of testing the butter during storage at 4 °C (storage duration test) showed the butter produced was of high microbiological quality (Tables 2 and 3). Pathogenic bacteria were not detected in the analyzed samples of butter, which indicates good manufacturing and good hygienic practices were applied during the butter manufacturing process. The resultant butter product was microbiologically safe and fit for human consumption.

In the 125 g packs of butter (Table 3), very small numbers of lipolytic bacteria were detected between 30 and 40 days of storage (2-11 cfu/g), while in the 20 kg blocks during the same timeframe, no lipolytic bacteria were detected. These lipolytic bacteria in the 125 g packs are the result, above all, of handling and the various actions that are performed before and during the packaging process and that lead to increased numbers/presence of these microorganisms. The 20 kg blocks of butter are much less handled than the smaller 125 g packs. The current study also confirmed the 125 g packs of butter contained molds, which were likely largely derived from their being airborne in the packaging hall (Table 1). As explained above, airborne contamination in production hall air could be directly incorporated in the butter via churning. The maximum number of molds determined in 125 g packs of butter during 60 days storage was 89 cfu/g, while in 20 kg blocks of butter, that number was lower and amounted to 22 cfu/g.

The results obtained showed airborne mold contamination was greater in the packaging hall than in the production hall housing the butter churning process [17]. Butter packed in 125 g portions are riskier forms of commercial packaging than the 20 kg blocks of butter as a result of various handling operations carried out before and during the machine packing. Also, the molds in the 125 g packs of butter were largely derived from the air in the packaging room, and these molds were likely directly deposited on the butter surfaces.

#### 4. Conclusion

Butter producers should conduct microbiological examination of final product as part of controls to determine product safety and appropriate expiration dates. Butter producers are required to have appropriate microclimate conditions (temperature and humidity), to document potential sources of microbiological contamination of air, and to safeguard against air contamination in order to obtain microbiologically acceptable products. Personnel hygiene and aspects of the premises' construction should be addressed, while special projects utilizing performance aeromicrobiology could be

implemented to solve any specific problems arising. Finally, methods of purifying air (conditioning, filtration, laminar flow, etc.) must be considered.

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# Safety of milk and whey from Zlatibor region in relation to aflatoxin M<sub>1</sub> contamination: a seasonal study

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**Abstract.** The objectives of this study were to assess aflatoxin M<sub>1</sub> (AFM<sub>1</sub>) contamination in cow and goat milk and whey samples collected from small dairy producers in rural areas of Zlatibor. The study involved a total of 60 samples of cow milk (n=15), cow whey (n=15), goat milk (n=15) and goat whey (n=15). In order to elucidate the distribution and stability of AFM<sub>1</sub> during milk processing, cheese manufacturing trials were performed using cow milk naturally contaminated with different concentrations of AFM<sub>1</sub> (<0.05 to >0.25 µg kg<sup>-1</sup>). AFM<sub>1</sub> concentrations were measured using a validated immunoassay method (ELISA). None of the samples of milk or whey collected from areas of Zlatibor were contaminated with AFM<sub>1</sub>. Contrary to that, in all of the analysed samples of whey obtained from contaminated cow milk, AFM<sub>1</sub> was detected. We conclude that whey contains 40-60% of the amount of AFM<sub>1</sub> present in milk. The results obtained indicate the intensification of dairy production and the supplementation with commercial feed such as maize could be risk factors that impact on the AFM<sub>1</sub> incidence in contaminated milk. In addition, our study demonstrates there is a potential health risk due to the consumption of whey obtained from contaminated milk.

## 1. Introduction

Milk and dairy products are very important in the human diet because of their positive influence on human health and ability to reduce the risks of cardiovascular disease, along with, particularly, the activity of conjugated linoleic acid (CLA) in inhibition of cancer, atherosclerosis and improvement of immune functions [1]. The dairy industry in Serbia is of great economic importance. According to official data, total milk production in Serbia is around 1506 million L/year (3505 L/per milked cow), with 97% of production being cow milk [2]. Thus far, cow milk is the most important type in Serbia, making up 96% of milk in the human diet, while goat and sheep milk production takes place on a small scale, and most of the milk is used by households or families.

Goat milk and its products are superior to cow milk, because goat milk is an excellent source of nutrients and is rich in high biological value proteins, essential fatty acids, high mineral bioavailability, volatile compounds (flavours, terpenes) and vitamin content [3]. Another aspect of the beneficial health effects of goat milk over other dairy species milks derives from the lower allergenicity of their proteins, greater digestibility and more bioactive components, meaning it is considered as a functional food [4]. Effort to encourage production and scientific research in this field will be positive for the goat dairy industry and for the commercialization of its products. Whey is a clear solution, a by-product of cheese



production. Whey is excellent source of nutrients including lactose, protein and minerals, but is often discarded or given to animals as nutritional supplement. However, it is used in specialized nutrition for athletes, body builders, elderly people, and people with obesity.

The hottest and driest period since 2010 in most of Serbia coincided with the most important growing phases of spring crops, causing substantial damage and losses in agricultural crop production manifested by high concentration of aflatoxins (AFs) in maize and, consequently, in feed and milk [5,6]. AFM<sub>1</sub> is the most significant toxin in the dairy industry because its water-soluble compounds tend to bind to the protein fraction of milk, it is very heat-stable, and thus, it can contaminate dairy products, which usually contain a higher concentration of AFM<sub>1</sub> than the milk from which it was made [7].

AFs are one of the major etiological factors in the development of hepatocellular carcinoma (HCC). Although, the toxic effect of AFM<sub>1</sub> is approximately 10-fold less than AFB<sub>1</sub>, AFM<sub>1</sub> has been classified as a Group 1 human carcinogen by the International Agency for Research on Cancer [8]. For this reason, and taking into account the significance of milk and milk products in the human diet (especially for children), the maximum permissible levels (MPL) of AFM<sub>1</sub> are strictly regulated worldwide. Serbia has set a MPL of 0.25 µg kg<sup>-1</sup> [9] which is between the MPL of the European Union (0.05 µg kg<sup>-1</sup>) [10] and the internationally recognized limit (0.5 µg kg<sup>-1</sup>) [11]. At the moment, this limit has been established following the ALARA (as Low as Reasonably Achievable) principle. Despite the fact that AFM<sub>1</sub> could also be 2-4 times higher in cheese than the levels initially present in milk [12], no specific AFM<sub>1</sub> limits have been set by the Serbian government for dairy products.

Serbia has also recorded a high estimated annual global burden of HCC cases attributable to AFM<sub>1</sub> exposure (0.01730.019 for males versus 0.0166-0.018 for females) [13]. There is a scarcity of systematic studies assessing the risk of exposure to AFM<sub>1</sub>-contaminated milk and dairy products such as whey, and that is coupled with the fact that AFM<sub>1</sub> contamination of milk has been a serious problem in Serbia in recent years due to the climatic conditions. Therefore, the aims of the present survey were to: 1) investigate the occurrence of AFM<sub>1</sub> in raw cow, goat and whey milk samples collected from small dairy producers from a mountain (Zlatibor) region, and; 2) assess the safety aspects, particularly due to whey consumption. This survey could be a useful scientific reference for the design of risk-based food safety surveillance and management for regional dairy production.

## 2. Materials and methods

### 2.1. Sample collection and preparation

The research was conducted during the autumn-winter season of 2018/2019 in Zlatibor region (South-Western Serbia 43° 45' 0.048" N and 19° 42' 56.023" E, 1,000 meters above sea level). During the study period, a total of 60 cow and goat milk and whey samples were collected from small dairy producers in rural areas of Zlatibor. In the autumn-winter season, all farms were feeding dairy stock mainly with haylage. Representative milk samples collected in plastic containers were transported under cold conditions to the laboratory of the Institute of Meat Hygiene and Technology and stored at -18°C until analysis. In order to elucidate distribution and stability of AFM<sub>1</sub> during milk processing, cheese manufacturing trials were performed from cow milk naturally contaminated with different concentrations of AFM<sub>1</sub> (<0.05 to >0.25 µg kg<sup>-1</sup>). The AFM<sub>1</sub> content in raw cow milk and whey were determined as previously described [13]. Samples were centrifuged for 10 min at 3500 g at 10 °C. After centrifugation, the upper cream layer was completely removed by aspirating through a Pasteur pipette. Skimmed milk was used directly in the test (100 µl per well).

### 2.2. Enzyme-linked immunosorbent assay (ELISA) analysis

The ELISA test procedure was performed using the Aflatoxin M1 ELISA kit (Tecna S.r.l., Italy). Preparation of the samples and ELISA test procedure were performed according to the instructions provided by the manufacturer. The detection limit of the method (LOD) was 0.005 µg kg<sup>-1</sup>. In the case of AFM<sub>1</sub> levels higher than 250 µg kg<sup>-1</sup>, samples were diluted with sample dilution buffer and reanalyzed. Relative standard deviation of reproducibility was 6%. Recovery was 110%.

### 3. Results and discussion

The occurrence of AFM<sub>1</sub> contamination in raw cow and goat milk samples and in whey collected from small dairy producers in rural areas of Zlatibor are presented in Table 1. Concentrations of AFM<sub>1</sub> found in each whey sample and naturally contaminated milk from which whey was made are presented in Table 2 and Figure 1.

#### 3.1. Occurrence of AFM<sub>1</sub>

Based on our results, concentrations of AFM<sub>1</sub> in all cow and goat milk and whey samples were below the LOD. Therefore, the contamination level of AFM<sub>1</sub> is not considered a public health concern under Serbian or EU legislation. These results show a significantly different situation compared to the previous study from Serbia [13,14]. Increased levels of AFM<sub>1</sub> in Serbian milk since 2013 were most probably the consequence of feeding corn contaminated with AFB<sub>1</sub> [15]. It is considered that pasture-based dairy production systems (as occurred in our current study) present low risk [16], because AFs are not produced in pasture, are rarely found in forages and are usually not present in high enough concentrations in corn silage to be of concern. Indeed, the risk of AFs occurring in milk is believed to be largely confined to the feeding of grain-based concentrates. However, the carry-over rates of AFB<sub>1</sub> in milk depend on the animal species, but also can vary greatly depending upon nutritional, environmental, and physiological factors such as stage of lactation, systemic diseases and local (mammary) infections, level of AFB<sub>1</sub> in feed, rate of feed ingestion, and geographical and seasonal conditions [17].

**Table 1.** The occurrence of AFM<sub>1</sub> in raw milk and whey from Zlatibor region

Type of milk		N	Incidence n (%)
Cow	Milk	15	0/15 (0)
	Whey	15	0/15 (0)
Goat	Milk	15	0/15 (0)
	Whey	15	0/15 (0)
Total		60	0/60 (0)

N – total number of analysed samples; n – number of positive samples

#### 3.2 Distribution and stability of AFM<sub>1</sub> during processing

Our study found there was a significant linear relationship between the amount of AFM<sub>1</sub> in contaminated milk and the AFM<sub>1</sub> concentration in whey (Fig. 1). During cheese manufacturing trials, 1L of cow milk produced 0.6 L of whey. The regression analyses between AFM<sub>1</sub> concentration in milk and whey show the AFM<sub>1</sub> concentrations in whey strongly depended on the AFM<sub>1</sub> concentrations in milk, and this is a good predictor of the AFM<sub>1</sub> concentration in whey. Most studies have reported that thermal treatments such as pasteurization and sterilization cannot change the level of AFM<sub>1</sub> in dairy products [18]. Based on the results of Škrbić et al. [19], the level of AFM<sub>1</sub> in cheese depends on several factors including type of cheese, cheese-making procedures, ripening conditions (e.g., temperature, humidity and pH) and contamination level of milk. Moreover, the distribution of AFM<sub>1</sub> between curd and whey can be variable.

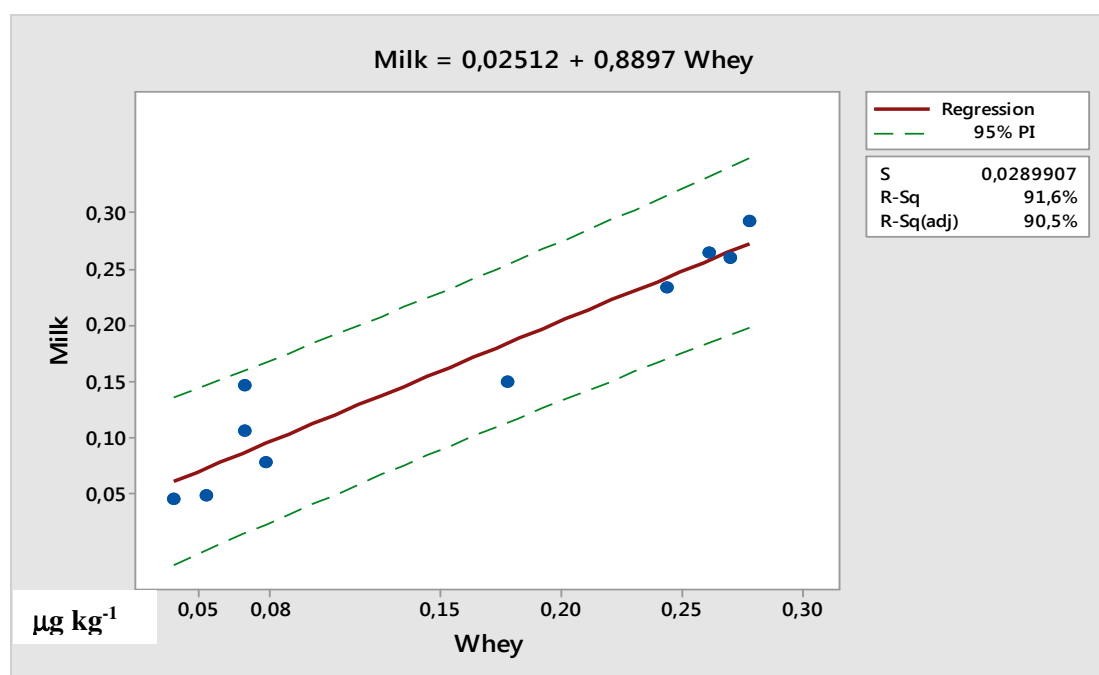
Our findings are in agreement with results of previous studies which concluded that during cheese production, 60% of the initial content of AFM<sub>1</sub> accumulates in the whey, while 40% of the AFM<sub>1</sub> remains in the curd or fresh cheese [12,17]. Accordingly, whey and cheese could be considered as one of the most important sources of AFM<sub>1</sub> among milk-based products. Beside milk, the presence of AFM<sub>1</sub> in other dairy products and the high intake of these products by the human population may have negative health implications for consumers. Based on the regulations of the EC and Codex Alimentarius

Commission, the allowed MPL for AFM<sub>1</sub> in dairy products is 0.250 µg kg<sup>-1</sup> [20,21]. Except for the level regulated in milk, there is no MPL for AFM<sub>1</sub> contents in dairy products in Serbia.

**Table 2.** Concentrations of AFM<sub>1</sub> in whey and naturally contaminated cow milk from which whey was obtained

Trial*	Milk		Whey	
	µg kg <sup>-1</sup>	Total AFM <sub>1</sub> (µg)	µg kg <sup>-1</sup>	Total AFM <sub>1</sub> (µg)
1	0.04	0.11	0.04	0.06
2	0.05	0.12	0.05	0.08
3	0.08	0.20	0.08	0.12
4	0.11	0.27	0.07	0.10
5	0.15	0.37	0.07	0.10
6	0.15	0.38	0.18	0.27
7	0.23	0.59	0.24	0.37
8	0.26	0.65	0.27	0.40
9	0.26	0.66	0.26	0.39
10	0.29	0.73	0.28	0.42

\*Note: 2.5 litres of milk naturally contaminated with AFM<sub>1</sub> was used



**Figure 1.** Relationship of AFM<sub>1</sub> concentration in whey and that in milk from which the whey was obtained

#### 4. Conclusion

In the whole milk chain, AFM<sub>1</sub> carry over from feed to milk and transfer from milk to dairy products can be a potential threat both for farmers and dairy producers, who need to avoid, in milk, AFM<sub>1</sub> contamination that exceeds the legally permitted limit and to estimate the expected AFM<sub>1</sub> concentration in dairy products made from contaminated milk. The results of our cheese manufacturing trials confirm the AFM<sub>1</sub> concentration in whey is strongly dependent on the AFM<sub>1</sub> concentration in milk and could be

40-60% of the level initially present in milk. Moreover, the AFM<sub>1</sub> concentration in milk can be a good predictor of AFM<sub>1</sub> presence in the final products. Despite these encouraging results of investigation of milk and whey from the pastoral Zlatibor region, the presence of AFM<sub>1</sub>, especially in cow milk, should never be underestimated or neglected, due to the unpredictability of climatic conditions.

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# Food packaging and modified atmosphere – roles, materials and benefits

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**Abstract.** Packaging is an integral element of the system that brings safe food to consumers. Although packaging has sometimes been viewed as an inconsequential act of inserting food into a film or box, this attitude has been a prime source of product failure. Meat packaging is the most dynamic area of meat technology today and it continues to configure the future of this branch of food industry. Retail meat packaging should fulfil specific technological and hygienic demands, as well as demands such as attractive packaging appearance, appropriate meat colour, consumer acceptability, etc. Consumers are also very sensitive with regards to the use of food additives in the food industry. During the last two decades, modified atmosphere packaging has become a dominant retail meat packaging technology. The purpose of this technology is to prolong the shelf life of foodstuffs by preventing or inhibiting biochemical reactions (fat oxidation, metmyoglobin formation), growth of spoilage bacteria and degree of product respiration. This technology is opening up new markets and offers the possibility of successfully establishing new products and thus extending product ranges. This paper critically reviews the most important aspects of packaging foodstuffs in modified atmosphere.

## 1. Introduction

Effective packaging begins with an understanding of the requirements of the product and its marketing. These needs can then be logically connected to materials and machines to create a packaging system. A study of a broad range of food product requirements and packaging can be a stimulus to applying more profitable, optimal packaging to food. By any measurement, packaging is a large industry in size, diversity and complexity. Mere enumeration of the amount of materials consumed or money volume of machinery sold would fail to account for the numbers of persons who package products each day. Entire industries are, in reality, not manufacturers but packagers of products made by or assembled by others. When food packaging is the topic at hand, the impact of packaging on shelf life, which is a very important factor in food industries, must be considered [1].

The shelf life of perishable foods as meat, poultry, fish, fruits and vegetables and bakery products is limited in the presence of normal air by two principal factors – the chemical effect of atmospheric oxygen and the growth of aerobic spoilage microorganisms. These factors both individually or in association with one another bring about changes in odour, flavour, colour and texture leading to an overall deterioration in quality. Chilled storage will slow down these undesirable changes but will not necessarily extend the shelf life sufficiently for retail distribution and display purposes. Food spoilage is defined as changes that make a product unacceptable for human consumption. Such changes include visible bacterial growth, slime formation, physical damage or off-odour. The process collectively known



as food spoilage is a very complex event, in which a combination of microbial and biochemical or chemical activities interact.

Packaging of food now performs beyond the conventional protection properties and provides many functions for the contained product [2]. During the last two decades, modified atmosphere packaging (MAP) has become a dominant retail meat packaging technology. The main factors stimulating MAP development are the continuous increase in consumption of fresh meat, an increase of urban populations and exhaustion of natural food resources. Developments in packaging materials and technologies have made the application of MAP on a larger scale to meat and meat products feasible [3]. Packaging a perishable product in an atmosphere that has been modified so its composition is other than that of air is termed as MAP. This refers to a system where the normal atmosphere, assumed to be approximately 78% N<sub>2</sub>, 21% O<sub>2</sub>, and <1% CO<sub>2</sub>, is intentionally changed to some other identified gas composition.

The main purposes of MAP of meat and meat products and other foodstuffs, are two-fold: to ensure the microbiological shelf life and the sensory quality of the product, including the colour, door and palatability. Many meat packaging systems currently exist, each with different attributes and applications. These systems range from overwrap packaging for short-term chilled storage and retail display, to a diversity of specified MAP systems for longer-term chilled storage and display, to vacuum packaging, bulk gas flushing or MAP systems using 100% carbon dioxide for long-term chilled storage. Preservation using MAP has been known for more than 100 years, but it was not commercially used until the latter part of the 20<sup>th</sup> century [4]. MAP was first used to extend the shelf life of apples by storing them in atmospheres with reduced oxygen and increased carbon dioxide concentrations. In the 1930s, MAP was used to transport fruit in the holds of ships. However, the technique was not introduced commercially for retail packs until the early 1970s. MAP techniques are now used on a wide range of fresh or chilled foods, reflecting the increase in consumer demand for longer shelf life foods and less use of preservatives. Through the use of natural gases and adequate packaging materials and machines, the quality of foodstuffs is maintained and their shelf life enhanced. During recent decades, MAP of various food products has been well studied and documented [5,6].

## **2. Gases used in MAP technology**

Although gases such as nitrous and nitric oxides, carbon monoxide, sulphur dioxide, ethylene, chlorine, ozone and propylene oxide have been investigated for use in MAP, they have not been applied commercially due to safety, regulatory and cost considerations.

### *2.1. Oxygen*

Oxygen has an important role in MAP, especially in packaging of fresh meat [5]. The colour of fresh meat is determined by the condition of myoglobin in the meat. When an anaerobic atmosphere is applied, myoglobin is transformed to metmyoglobin, producing a brown colour that is undesirable to consumers. It is, therefore, essential that oxygen is included in the applied gas atmosphere when fresh meat is packaged. This will ensure the myoglobin is oxygenated, resulting in an attractive bright red colour [6]. Oxygen is a fairly active molecule and is associated with the process of the oxidation, i.e. the change of the chemical state of some biological molecules. The chemical breakdown of lipids is the primary degradation process in dry or in dehydrated foodstuffs and in high fat fish. This is due to the oxidation of unsaturated fats in the presence of atmospheric oxygen, causing the product to turn rancid. Reduced oxygen concentration within the package can prevent or slow down oxidative reactions such as lipid rancidity in meats, fish and bakery foods, which would result in off odours and flavours, or the browning reaction in cut fresh fruits due to the action of polyphenol oxidase. However, complete absence of oxygen is not desirable either. For example, the gaseous mixture used for fresh meat usually contains 80% oxygen in order to maintain the fresh bright red colour.

### *2.2. Carbon dioxide*

Carbon dioxide is the most important gas in the field of MAP technology. Carbon dioxide is a quite active gas as opposed to the inertness of nitrogen. Carbon dioxide can inhibit the growth of several types

of microorganisms, especially those that cause slime and off-odours in refrigerated foods. This gas is both water- and lipid-soluble and although it is not a bactericide or fungicide, carbon dioxide has bacteriostatic and fungistatic properties. The bacteriostatic and fungistatic properties of carbon dioxide have been widely recognized since the 1920s, when it was used in shipments of beef, mutton and lamb from Australia and New Zealand to England. Its solubility increases with decreasing temperature and higher food pH. The precise mechanism of carbon dioxide action is still a subject of considerable interest and is not as well understood as mechanisms of other external factors acting in food, such as water activity and pH. What usually happens to perishable products stored in elevated levels of carbon dioxide is a change not only in the numbers of microorganisms, but in the types of organisms present. Very often, this shift is from Gram-negative genera to Gram-positive bacteria such as *Streptococcus* and *Lactobacillus* [7]. The intensity of carbon dioxide activity depends on concentration of gas, initial bacterial contamination of foods, storage temperature and nature of packaged food [8,9]. Carbon dioxide also has the advantage that it is relatively nontoxic to humans. If carbon dioxide is part of a modified atmosphere where its bacteriostatic effect is desired, the minimum concentration of this gas should be 20%.

### 2.3. Nitrogen

Nitrogen has been used in MAP for many years due to its inert property. It is an inert, tasteless gas. Nitrogen can displace oxygen in MAP, thus extending food shelf life. It prevents fat rancidity and inhibits the growth of aerobic microorganisms. Moreover, use of nitrogen in MAP can prevent package collapse due to this gas's low solubility in both the water and fat phases of foods [10]. The gas has no direct effects on colour of food. Also, the role of nitrogen in MAP is to act as filler gas and keep flexible packages from developing a vacuum [11].

## 3. Packaging materials

The optimisation of packaging is a decisive factor for the efficiency of MAP. The packaging must have appropriately low oxygen/gas permeability and tight seals, otherwise too much gas can penetrate. Packaging materials, primarily polyamides, have the potential of not only approaching the desirable properties of animal and other natural casings, but also of serving as a basis for new achievements in food packaging applications. This is largely due to the analogies and similarities between the physical and chemical properties of polymers, especially those of polyamides and biopolymers. Only the great progress in chemistry and technology after the Second World War enabled serious developments in the manufacture of more sophisticated synthetic polymer materials, generally used for packaging high volume food items. New, alternative packaging materials, production methods, novel applications and permanent innovations of existing materials and ways to use them are continually being investigated and verified by the food processing industry in the pursuit of producing better quality products, more economically and more successfully.

The production and use of polymer materials require knowledge from various and diverse sources to understand possible utility or validity of the materials, and polymer use aims to create new, technologically more satisfying and economically more acceptable food products. Such an eclectic approach starts with the development of polymer processing technology and ends with complex food production systems, including numerous safety, technical, technological, environmental, nutritional, biochemical and other advancements.

The chemical nature of monomer units, methods of polymerization (synthesis), length of chain formed (molecular mass), and intermolecular forces within the polymer chain are deciding factors in determining many polymer processing properties. The properties of different polymer macromolecules are determined by the chemical nature of monomer groups and the length of chains. The chemical nature of the monomer unit and its functional groups determine polymer toughness, stiffness, transparency, gas and water vapour permeability and other basic properties of the polymer. Especially, the polymer resistance to deformation, its rigidity and its barrier properties depend largely on the polarity of the polymer pendant group.

Numerous man-made polymers are used for the manufacture of packaging materials that are in use for MAP. They are polyethylene (PE), ethylene vinyl acetate copolymer (EVA), ethylene vinyl alcohol copolymer (EVOH), polypropylene (PP), polyvinylidene dichloride (PVDC), polyethylene terephthalate (PET), polyamides (PA) and some more.

Polyethylene (PE) is the most common polymer used in food packaging. It has good sealing properties and is of relatively low cost. Polyethylene is formed by the addition polymerization of ethylene gas. Polyethylene exhibits good resistance to chemicals, it has good moisture barrier properties, but it is a poor barrier for oxygen and many organic chemicals. Depending on the reaction conditions, three types of polyethylene are produced: low density (LDPE), high density (HDPE) and low linear density polyethylene (LLDPE).

Ethylene vinyl acetate (EVA), the most common and cheapest copolymer of ethylene, is widely used as adhesive in coextrusion, for example, between polyamide and polyethylene layers. It has similar properties to PE, but much greater toughness.

Ethylene vinyl alcohol (EVOH) is a copolymer of ethylene and vinyl alcohol. EVOH has increasing application as the oxygen barrier layer in multilayer polymer films and casings.

Polypropylene (PP) is widely used in manufacturing fibres, packaging films and moulded and extruded articles.

Polyvinylidene dichloride (PVDC) is a crystalline thermoplastic polymer of vinylidene dichloride. It can be formed into waterproof and chemically resistant filaments, films, fabrics, etc.

Polyethylene terephthalate (PET) is the most important polymer in the class of polyesters. PET is the polycondensation product of terephthalic acid and ethylene diol. PET has excellent mechanical strength. It is a heat-stable crystalline polymer with a high melting point of 258°C to 262°C.

Polyamides (PA) include a very broad group of polymers used as fibre, engineering plastics, adhesives, inks, coatings, films and in other applications requiring high heat and chemical resistance.

Some of the most important properties of the polymer films are shown in Table 1.

**Table 1.** Important properties of different 25 µm-thick polymer films [2]

		PE	EVA	PP	EVOH	PVDC	PET	PA6
Melting point	°C	100-140	85-110	150-165	176	158	260	220
Tensile strength	MPa	23-35	10-20	30-40	25	100	100	80-90
Tensile modulus	GPa	100-300	50-120	1500-3000	1000-2000	800	2000	1500
Elongation at break	%	500-800	600-900	250-600	150	90	250-600	300-500
Water vapour transmission	g/m <sup>2</sup> d	0,5-5	2,10	1-5	50	2-5	15	40
Oxygen transmission (dry)	ml/m <sup>2</sup> d bar	4000	5000	2500	0,1-1	4	160	40

Oxygen	ml/m <sup>2</sup> d	4000	5000	2500	3-10	4	160	80
transmission	bar							
(85% relative								
humidity)								

PE, polyethylene; EVA, ethylene vinyl acetate copolymer; EVOH, ethylene vinyl alcohol copolymer; PP, polypropylene; PVDC, polyvinylidene dichloride; PET, polyethylene terephthalate; PA6, polyamide 6.

Polyamides show a variety of different properties, potentially useful for sausage casing production. It has been already emphasized that they have impressive mechanical and thermal resistance, allowing their use as engineering thermoplastics. In addition, many of these properties, such as melting point, toughness, and resistance to cracking and other stresses can be modified and tailored by adequate combinations of aromatic and long-chain aliphatic dicarboxylic acids with bulky cycloaliphatic diamines.

Polyamides absorb considerable amounts of water. The amount of absorbed water depends on relative humidity and temperature of environment, the exposure time, polyamide type and its crystallinity and thickness of the film. Water is inserted into the hydrogen bonds, weakening these strong intermolecular forces and reducing strength and stiffness of polyamides, while increasing their flexibility and elasticity. However, migration of water into polyamide is a relatively slow process. Microcrystalline polyamides are milky white in colour and differ from transparent polyamides that are characterized by very low or non-existent crystallinity. Most of the semicrystalline aliphatic polyamides are transparent in film thicknesses below 0.5 mm. The properties of transparent polyamides are little affected by temperature. Light transmission can be reduced by addition of particulate additives. The transparency of polyamides can be improved by increasing the number of spherulites through addition of nucleating agents: fine-grain crystallites of average diameter below the wavelength of visible light will provide highly transparent film.

Polyamide films are used in food packaging both in single layer and in multilayer structures with other materials (polyethylene copolymers, polyethylene, polyvinylidene chloride, etc.). The multilayer structures are produced by coextrusion of the two or more plastic materials.

#### 4. Conclusion

The use of MAP in food packaging has been practiced for about 100 years, but the potential that can be achieved using this technology has still not been realized completely. MAP, if used properly for the right commercial reasons, offers sufficient benefits to both food industries and to consumers. This suggests MAP is one of many alternatives that industry should consider and use as part of applied marketing programs for high quality food. Only the highest quality food products should be used to benefit from the extended shelf life advantages afforded by MAP. The use of this technology to make up for defects in product quality and limitations in transportation will only lead to consumer dissatisfaction. The use of MAP does not eliminate the need for proper control of storage conditions, especially temperature, nor for the adequate training of food handlers at every stage of the food preparation process.

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# Screening of *Bacillus cereus* presence in minced meat and meat products originating from Serbian retail facilities

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**Abstract.** The goal of this study was to investigate the prevalence and numbers of *B. cereus* in minced meat and meat products. Eighty-seven meat or meat product samples were collected from different retail facilities in Serbia. Meat samples were subjected to microbiological analyses using the conventional cultural method for counting and isolation of *B. cereus*, confirmed by microscopic and biochemical tests. Eighty-seven samples included raw minced meat (7), semi-processed meat (60), processed meat (7) and meat products (13). The prevalence of *B. cereus* in the collected samples was 34.5 % (30/87). This pathogen was not isolated from raw minced meats, unlike semi-processed meat products, where it had a prevalence of 40 % (24 samples). Among processed meats, 28.6 % (2) were positive, while in meat products, the *B. cereus* prevalence was 30.8 % (4). We hope these results will provide better understanding of *B. cereus* isolated from food of animal origin in Serbia and results indicate meat and meat products might play an important role in dissemination of this pathogen through the food chain.

## 1. Introduction

All over the world, a major cause of mortality is reported to be foodborne diseases, which are a serious threat to public health. Rapid economic development, mass food production, population growth, increasing trans-border movement of people, animals and animal products, etc. play significant roles in the increasing number of outbreaks throughout the world. The main problem is that the greatest number of cases of foodborne disease goes unnoticed because symptoms are often mild, with associated diarrhoea and vomiting, and as such, they remain undiagnosed.

*Bacillus cereus* was identified as a causative agent of foodborne illness in the 1950s. This member of the family Bacillaceae is a gram-positive, rod shaped, aerobic, motile and  $\beta$ -haemolytic bacterium. The vegetative cells are typically 3.0-5.0  $\mu\text{m}$  by 1.0-1.2  $\mu\text{m}$  in size. It is capable of forming ellipsoid-shaped spores in a central or paracentral position without swelling the sporangium. In addition, this bacterium can grow in a broad pH range of 4.9-9.3, with the minimum pH 4.35 for growth in meat [1][2]. It is frequently isolated from soil, but also from food production environments, foods and intestinal tracts of insects and mammals [3][4].

Foodborne outbreaks of *B. cereus* have been reported by many countries, as in Hungary, where it has been found to be the third most common cause of food poisoning cases. Furthermore, the presence of *B. cereus* as a meat contaminant was reported by some investigators, not only in raw meat but also in meat products [5][6]. Consequently, *B. cereus* is also considered as an important pathogen in heat-treated foods [7]. In addition, *B. cereus* has been isolated from the stools of healthy adults and children (43 %) in various concentrations [8].

This pathogen causes two distinct types of food poisoning – diarrhoea and emesis – caused by two different types of toxins [9]. The first form of illness is characterised by abdominal pain and diarrhoea, which occurs between 8 and 16 h after ingestion of the contaminated food. The second form is characterised by nausea and vomiting, which happens within 30 min to 5 h after eating contaminated food [10]. The diarrheal form of disease is associated with three types of enterotoxins: three-



component non-haemolytic enterotoxin, three-component enterotoxin hemolysin BL and the single-component enterotoxin cytotoxin K [11]. Diarrheal toxins are produced by the bacteria during their growth in the small intestine. Unlike the diarrheal form, emetic syndrome is caused by emetic toxin that is synthesized during the bacterium's growth phase in the food [12]. The infective dose of *B. cereus* in emetic food poisoning is  $10^4$  to  $10^{11}$  cfu/g or ml, while in diarrheal food poisoning, it is between  $10^5$  and  $10^{10}$  cfu/g [13], [14].

According to the report by EFSA BIOHAZ Panel's Working Group, almost all strains of *B. cereus* are resistant to  $\beta$ -lactam antibiotics due to the presence of beta-lactamase genes [15]. Bearing in mind these facts, the goal of this study was to isolate and enumerate *B. cereus* from minced meat and meat products of various animal species from retail facilities in Serbia and to get preliminary results which would be a guide for additional research in this field.

## 2. Materials and methods

### 2.1. Samples

Samples of meat and meat products were collected between January and March 2019 from various retail facilities in different locations in Serbia. Eighty-seven samples (Table 1) including 7 minced meat samples of different species (beef, pork and chicken), 60 semi-processed meat samples (burgers, raw sausages, marinated meats and ćevapi), 7 processed meat products (ready meals) and 13 meat products (raw fermented and boiled sausages and pates) were examined for the presence of *B. cereus*. All samples were collected into sterile containers, stored in an insulated icebox, and transferred in the shortest possible time to the laboratory.

### 2.2. Microbiological method of isolation and identification

Isolation and identification of *B. cereus* in our samples were performed following the SRPS ISO 7932:2009. *B. cereus* selective differential Mannitol Egg-Yolk Polymyxin (MYP) agar base (Park Scientific Ltd., UK) was used to isolate presumptive colonies. MYP plates were surface plated by spreading of 0.1 mL of appropriate serial dilutions of tissue homogenate and then incubated at 30°C for 24-48 h. MYP plates were checked for the presence of pink colour colonies surrounded by a precipitation zone of the same colour, which indicates that lecithinase is produced. Those plates which contained a countable range of 15-150 colonies were counted. The control strain in this study was *B. cereus* ATCC 11778 (Microbiologics Inc., St Cloud, MN, USA).

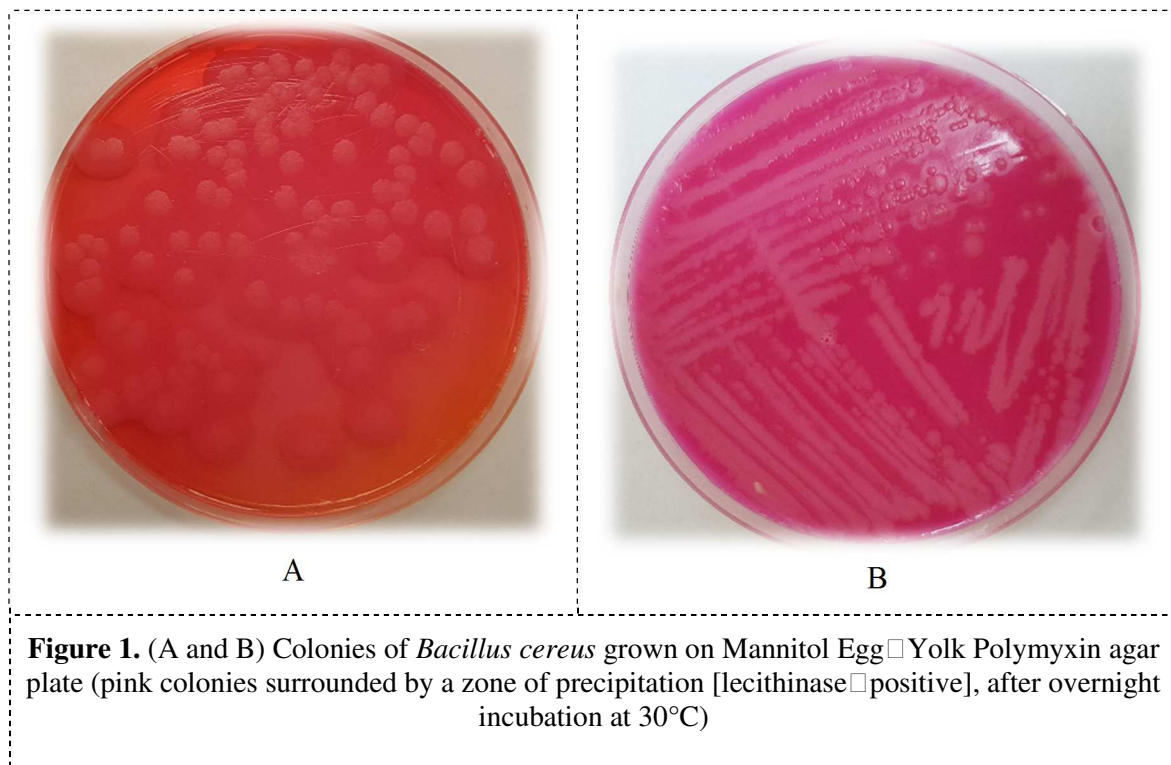
### 2.3. Confirmation of *Bacillus cereus*

Presumptive colonies were confirmed using morphological and biochemical tests. The gram stain procedure was used for microscopic examination, where *B. cereus* appeared as large gram-positive bacilli in both short and long chains. Furthermore, biochemical reactions typical for *B. cereus* were utilised for further confirmation (lecithinase, oxidase,  $\beta$ -haemolysis test, motility, sugar fermentation: arabinose, mannitol and xylose) [16].

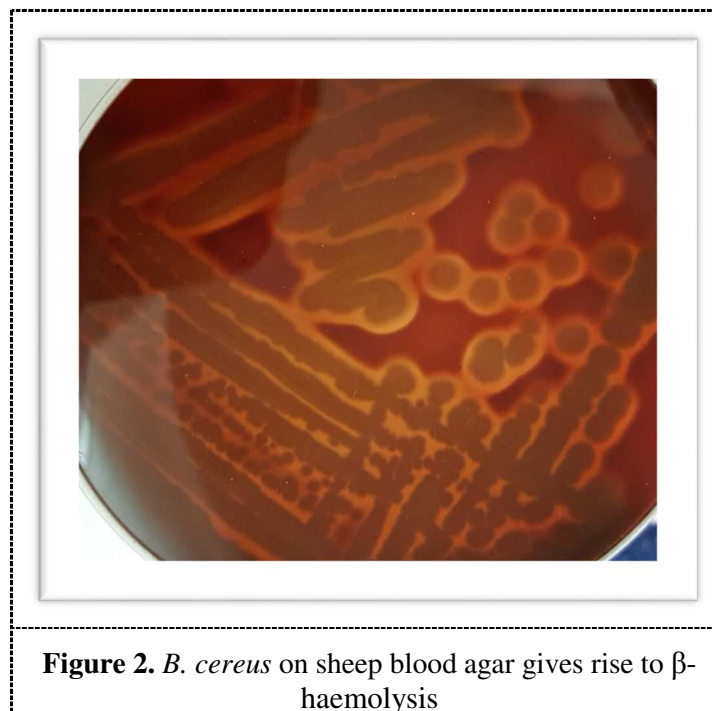
## 3. Results and discussion

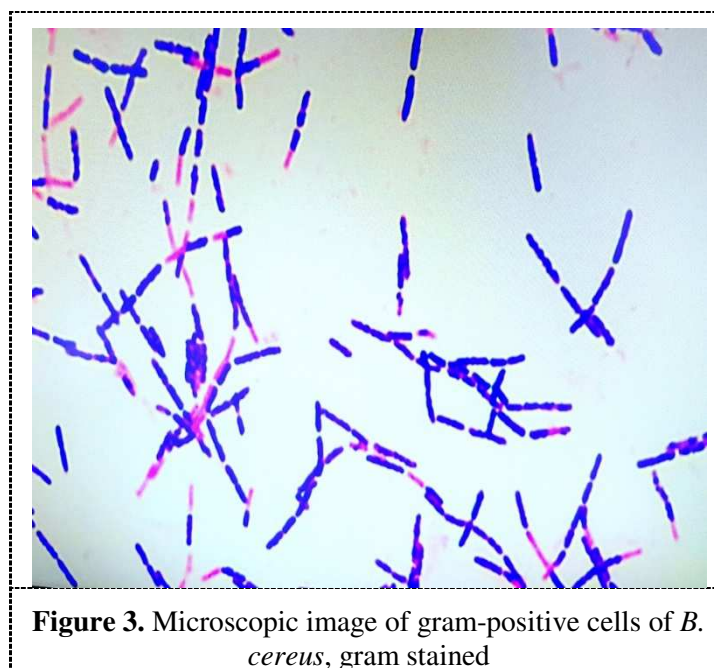
Eighty-seven retail meat samples (Table 1) from various locations in Serbia were tested for the presence of *B. cereus* using MYP medium. On MYP medium, 30 samples of meat products produced pink coloured colonies surrounded by precipitation zones, and which were presumed as *B. cereus* (Figure 1). Presumptive positive *B. cereus* colonies were sent on for further confirmation.





Every one of the 30 presumptive meat products produced colonies positive for sugar fermentation (arabinose, mannitol and xylose) characteristic of *B. cereus*. Oxidase and motility were also positive and  $\beta$ -haemolysis was produced by all of them (Figure 2). Furthermore, microscopic examination also confirmed positive results in the 30 presumptive meat products, since the bulky gram-positive bacilli in both short and long chains were typical of *B. cereus* (Figure 3).





In general, the prevalence of *B. cereus* in the collected samples was 34.5 % (Table 1). The pathogen was not isolated from minced meat, unlike semi-processed products where the prevalence of *B. cereus* was 40 % (24 samples). Numbers of *B. cereus* in positive samples ranged between  $2 \times 10^1$  cfu/g and  $4.9 \times 10^6$  cfu/g. Among 7 processed meat samples, 28.6 % (2) were positive, with levels ranging from  $1.7 \times 10^3$  cfu/g to  $2.0 \times 10^5$  cfu/g. Furthermore, in meat products, the *B. cereus* prevalence was 30.8 % (4), with levels ranging from  $2 \times 10^2$  cfu/g to  $8.2 \times 10^4$  cfu/g.

**Table 1.** Prevalence of *B. cereus* in meats grouped by processing category

Sample category	Number of tested samples	Number of positive samples	Prevalence (%)
Minced meat	7	0	/
Semi-processed meat	60	24	40
Processed meat	7	2	28.6
Meat products	13	4	30.8
Total samples	87	30	34.5

The results obtained in this study concurred with the results of Eldaghayes et al., who reported a prevalence of 29 % [17]. A similar prevalence (27.8 %) was obtained by Tewari et al. [18]. The prevalence in meat and meat products consumed in Turkey was 22.4 %, which is also close to our results [19]. Mudasir Bashir et al. [1] reported a prevalence of 29.33 % in their research in India. On

the other hand, Smykal and Rokoszewska conducted research over a 7-year period (1964-1971), where the prevalence of *B. cereus* was 13.3 % in meat and meat products [20]. The different (higher) prevalence in our study could be explained by poor hygiene and cross-contamination during production in our meat products. Our meat products could initially have been contaminated with larger numbers of microorganisms, meaning they were more prone to carry-over of these organisms during processing. However, it has been proved that the prevalence of *B. cereus* is often much higher in raw or undercooked products compared with cooked ones, because the absence of heating process limits reduction of microbial load [21]. Still, this study failed to detect *B. cereus* in the raw minced meats sampled. The reasons may be the low bacterial loads as well as the relatively small number of minced meats sampled. The largest number of tested samples belonged to the group of semi-processed meat products, and in this group, the *B. cereus* prevalence was also the highest. The main reason might be the fact that these are raw meats, but during the manufacturing processes, additional manipulation occurs. All these subsequent operations could lead to additional contamination. Furthermore, in this group of meat products, spices are also added, and spices are one of the main recognised sources of *B. cereus*. Still, the prevalence of *B. cereus* in semi-processed meat products was lower than reported previously [22]. The prevalences of *B. cereus* in cooked meats (30.8 %) and processed meats (28.6%) was lower than in semi-processed meats (40.0 %), which was anticipated since these former products are thermally treated [21]. Nevertheless, our prevalences in cooked meats were higher than results recorded by Eglezos et al. for cooked sausage rolls [23]. The *B. cereus* prevalence in our meat products was lower than that found by Smith et al. [24]. However, the relatively high prevalence of *B. cereus* in our meat products indicates there might be problems during production processes. These products are ready-to-eat, so they could be a high risk to consumers [25].

#### 4. Conclusion

The presence of *B. cereus* in meat products is an important aspect of food safety for consumers, especially for immunocompromised people. Of course, our research is just one piece of the puzzle and if we want to complete the whole picture, we must continue with extensive and detailed research. Nevertheless, we hope these results will provide a better understanding of the presence of *B. cereus* isolated from food of animal origin in Serbia. We believe the results indicate meat and meat products might play an important role in spreading *B. cereus* through the food chain. Generally, the high prevalence of *B. cereus* in meat products is associated with poor hygiene in abattoirs, contaminated additives and cross-contamination during preparation and storage. These findings surely demand strict implementation of good hygienic practices (GHP) and hazard analysis and critical control point (HACCP) procedures at all stages of the food chain, to decrease the prevalence and number of *B. cereus* and prevent its further dissemination.

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# Effect of virgin coconut oil on caecal microbiota composition in alloxan-induced diabetic rats

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**Abstract.** The gut microbiota is a complex community of a diverse population of obligate and facultative anaerobic microorganisms that could affect host metabolism and immune homeostasis. The effects of virgin coconut oil on the mean weekly fasting glycaemia, weekly body mass gains and daily water and food intakes after 16 weeks, as well as on the changes in composition of caecal microbiota in both non-diabetic and alloxan-induced diabetic rats, were investigated. The beneficial effects of virgin coconut oil were observed for all examined parameters. Additionally, this oil's potential to positively affect the caecum microbiome, with significant increase in the abundance of beneficial bacteria such as *Lactobacillus*, *Allobaculum* and *Bifidobacterium* species, was proven.

## 1. Introduction

Coconut (*Cocos nucifera*) is known as one of the most popular natural sources of short and medium chains fatty acids with lauric acid as dominant. Coconut oil is thought to have anti-obesity effects, and due to the presence of lauric acid, antimicrobial effects as well.

The microbiota of the intestine consists of a diverse population of obligate and facultative anaerobic microorganisms with a wide range of metabolic activities that provide essential nutrients for the host [1]. Consumption of particular types of food produces predictable shifts in the host's bacterial genera. There is a substantial body of evidence that dysbiosis or imbalance in gut microbiota is associated with various diseases, including colorectal cancer, liver cirrhosis, arthritis, atherosclerosis, ulcerative colitis, Crohn's disease, metabolic syndrome, allergy, asthma, eczema and autism [2-5]. Furthermore, the connection of type 2 *diabetes mellitus* (DM) and obesity with alterations in gut microbiome is well established. DM is a metabolic endocrine disorder resulting from a deficiency in insulin secretion and insulin action. Despite the large number of medicines for the treatment of diabetes, the complications caused by this disease remain a major medical problem [6]. Therefore, the search for oral herbal medicinal products for long-term blood glucose control in patients with DM is very important.

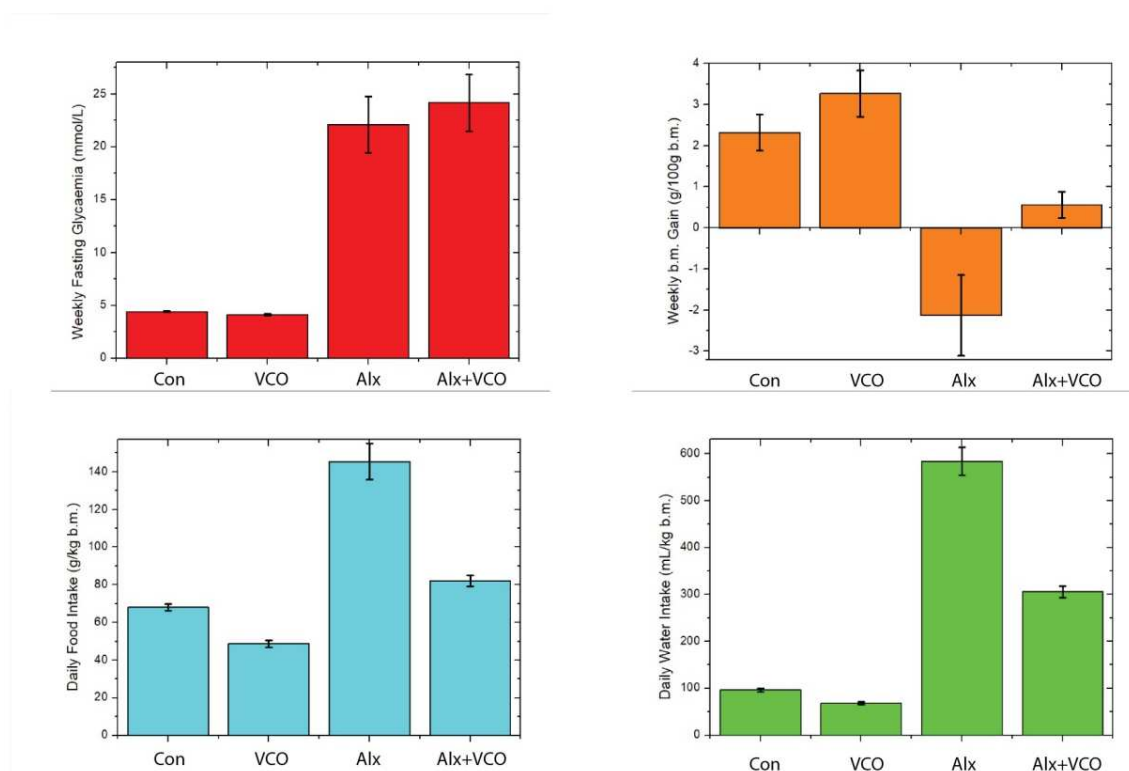
Consequently, in our recent work, we focused on determining the effects of *Cocos nucifera* virgin oil (VCO) on the caecum microbiota composition changes in both non-diabetic and alloxan-induced diabetic rats, as well as on determining mean fasting glycaemia, weekly body mass gains and daily water and food intakes [7]. Alloxan is widely used to induce DM in experimental animals, due to its generation of excess reactive oxygen species, which lead to destruction of pancreatic  $\beta$ -cells [8]. The main goal of this study was to present the most important findings of this research.





## 2. Physiological parameters

Four groups of rats, differing from each other by the combinations of alloxan treatment and coconut oil administration (Control (con), VCO, Alloxan (Alx) and Alx+VCO groups) were included in the investigation. We studied the glycaemic level by using tail fresh capillary whole blood samples and handy Wellion CALLA Light blood glucose test strips. In addition, body mass gain was measured weekly, while food and water intake was measured daily. The results obtained are presented in Figure 1.



\* b.m. - body mass; data are given as mean  $\pm$  standard error of the mean ( $\bar{X} \pm \text{SEM}$ ), with the  $p < 0.05$  as the minimal significant level. Significantly different: <sup>a</sup> in respect to Con; <sup>b</sup> in respect to VCO; <sup>c</sup> in respect to ALX. Control (con), virgin coconut oil (VCO), alloxan (Alx) and Alx+VCO groups.

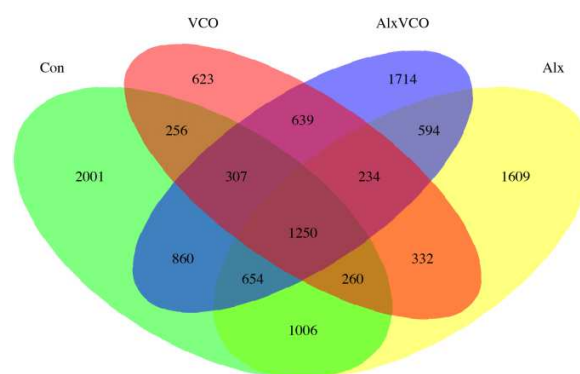
**Figure 1.** Mean weekly fasting glycaemia, daily food and water intakes and weekly body mass gains, after 16 weeks

Coconut oil supplementation significantly increased mean body mass gain, and lowered glycaemia, and food and water intake in the VCO group compared to the control. This effect was partly noticed in the alloxan-induced diabetes: there was a significant decrease in the food and water intake, and an increase in the body mass gain in the Alx+VCO group compared to Alx group. The beneficial effect of coconut oil supplementation in the Alx+VCO group lies in the fact that this oil is an energetically highly efficient food, with a high content of fatty acids that can be metabolized into metabolic water. The hypoglycaemic effect of coconut oil could be due to the presence of lauric acid, which has insulin tropic properties and the content of which is particularly high in VCO [9,10]. Furthermore, VCO polyphenols may enhance the

sensitivity to insulin and reduce insulin resistance and damage of pancreatic  $\beta$ -cells by scavenging reactive oxygen species [11, 12].

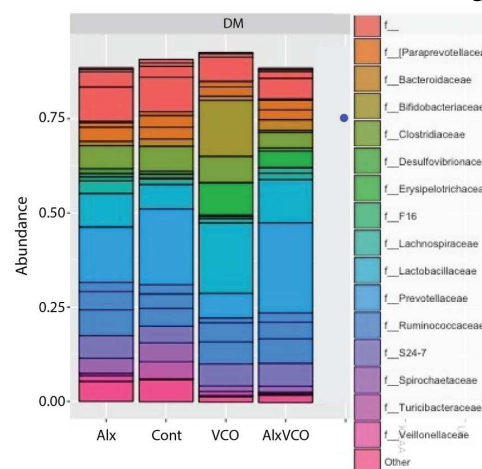
### 3. Caecal microbiota composition

The abundance and composition of the caecal microbiota of all tested groups was investigated in an *in vitro* study performed under the 16S rRNA NGS sequencing Illumina platform. The effect of VCO on intestinal community richness was also evaluated, and the maximum value of unique Operational Taxonomic Units (OTUs) was found in the control group, while the minimum value of OTUs was found in the VCO group. The overlap between the groups is shown in the Venn diagram (Figure 2), and it is noticeable that 1250 (13%) of the total 9614 taxa were divided among all groups.



**Figure 2.** Venn diagram, taken from [7], depicts the overlap of OTUs in caecal microbiota between the groups. Control (con), virgin coconut oil (VCO), alloxan (Alx) and Alx+VCO groups.

The structure of caecal microbiota at the phylum level showed the dominant bacteria in all rat groups were Gram-negative Bacteroidetes and Gram-positive Firmicutes, accounting for 77% to 90% of total bacterial rRNA-targeted sample sequences. At a family level, the caecal microbiomes of all four groups of rats demonstrated similar richness, but different bacterial abundances (Figure 3).



**Figure 3.** Percentage abundance at the family level in the caecal microbiomes of four rat groups. Only taxa with total percentage abundance above 0.5% across all samples were included. Alloxan (Alx), control (Cont), virgin coconut oil (VCO), and Alx+VCO groups

Particularly, coconut oil supplementation was positively correlated with family Lactobacillaceae (*Lactobacillus*) and Erysipelotrichaceae (*Allobaculum*). The effect of VCO on family Prevotellaceae, with detected *Prevotella* genus, was clearly dependant on the glycaemic status of the rats. In healthy animals, the oil radically decreased the abundance of this genus, but in diabetic animals, the effect was opposite. The effect on Bifidobacteriaceae (*Bifidobacterium* species) is worth mentioning, because an extremely high percentage of *Bifidobacterium* spp. was observed only in the VCO group. On the other hand, reductions of the number of bacteria from the Spirochaetaceae family, in this case, *Treponema* genus, were detected in VCO and Alx+VCO groups, while in the case of Turicibacteriaceae (*Turicibacter* species) a significant decrease in the abundance in the VCO group was observed.

In conclusion, we proved the beneficial effect of virgin coconut oil on some physiological parameters associated with diabetes in rats, i.e. food and water intakes and average body mass gain. In addition, the positive effect of coconut oil on the caecum microbiome, with significant increase in the abundance of beneficial bacteria *Lactobacillus*, *Allobaculum* and *Bifidobacterium* species, was detected. Additional research is needed to examine the variety of microbial species in the gastrointestinal tract and the diversity of microbial genes and their functions.

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## Physical properties (pH and $a_w$ value) of fermented sausages inoculated with *Yersinia enterocolitica*

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**Abstract.** Fermented sausages are produced without heat treatment, and the conservative effect is achieved by a combination of three factors: pH, lowering the water activity ( $a_w$ ) and the creation of lactic acid. Here, we summarize the results of the production of fermented sausages inoculated with *Y. enterocolitica*, with the added starter culture. Number of *Y. enterocolitica* declined during ripening, as did pH and  $a_w$  value. During the ripening process, the  $a_w$  of fermented sausages of narrower and wider diameters significantly decreased and was lower in sausages in which starter culture was used. At the end of the production process, there were no significant differences between the physical parameters of sausage quality (pH and  $a_w$ ) among fermented sausages with and without added starter culture, and between sausages with narrower and wider diameters.

### 1. Introduction

Different types of foods can be the cause of food-borne illnesses, and one of these is the zoonotic infectious agent, *Yersinia enterocolitica* [1]. Due to the importance of *Yersinia* in meat, the European Food Safety Authority (EFSA) recommended that is mandatory to examine pig carcasses for the presence of *Y. enterocolitica* [2].

The genus *Yersinia* belongs to the family *Enterobacteriaceae*. In this genus, three of the 12 species are pathogens, among them *Y. enterocolitica*, and six biotypes are recognised (five pathogenic and one non-pathogenic). This Gram negative, facultative anaerobe grows in a wide range of temperatures: optimal 28-29°C, minimum -2°C [3]. It grows in a wide pH range, 4.2-10 and at a minimum water activity ( $a_w$ ) of 0.96 [3, 4].

### 2. Fermented sausages

Fermentation of meat is based on the presence of lactic acid bacteria and lowering pH values. Further, starter cultures produce lactic acid [5], bacteriocins [6] and hydrogen peroxide [7], which act antagonistically in relation to pathogenic micro-organisms [5]. The use of starter cultures, designated for production of favorable microbiota, is to inhibit the growth of undesirable micro-organisms, as a result of fermentation of sugars into lactic acid [8]. Good sustainability of raw fermented sausages is based primarily on the  $a_w$  and pH value of the finished product. The water content of dry fermented



sausage is below 35%, but can be less than 30% in many cases, which corresponds to  $a_w$  of 0.90 or less and makes the product viable [9,4]. Traditional production of fermented raw sausage, in households in Serbia, takes place under uncontrolled conditions (temperature, humidity, and fermentation) during colder seasons. This process relies on the activity of fermentative bacteria that are naturally present in the meat and the production area environment [8]. Preservation of traditional production and authentic products can help small producers and local economies, but it is also important to preserve the traditional knowledge, cultural heritage and regional identity found in often small and underdeveloped locations [10-14].

### 3. *Yersinia*, pH and $a_w$ in fermented sausages

In central and northern Europe, the fermented reduction in pH (pH 5.6 to 5.8 drops to 4.6 to 4.9) is much more important for sausage preservation than in Mediterranean countries, where a significant reduction in  $a_w$  is more important [15]. In food with a neutral pH stored at 5 °C, there is a possibility that the number of *Y. enterocolitica* increase in a short period [16]. Minimum growth pH for *Y. enterocolitica* is between 4.2 and 4.4. *Y. enterocolitica* is not capable of growth at pHs below 4.2 or above 9. *Y. enterocolitica* has the potential to multiply during storage of meat and meat products. Storage temperature, vacuum packaging or packaging in a modified atmosphere can encourage bacterial multiplication [15,17]. The ability of *Y. enterocolitica* to survive at normal pH values is small, particularly at low temperatures [10-12]. According to the Ordinance on the quality of minced meat and semi-finished meat products, bacon should have pH of at least 5 [18]. The presence of an organic acid reduces the ability of *Y. enterocolitica* multiplying [15,17]. Usually, the  $a_w$  value of sausage filling or batter at the start of the production process is about 0.96 [19]. On processing,  $a_w$  continues to decline and the content of protein, fat, ash, and salts rises due to drying ([20,21], so if a starter culture is used, after four days of ripening,  $a_w$  is reduced from 0.97 to 0.94. After 12 days of ripening,  $a_w$  is between 0.89 and 0.90 [22,23]. However, there can be significant diversity in the raw materials, method of making sausages, fermentation and drying conditions. This results in different pH and  $a_w$  levels at the end of the manufacturing process, so individual fermented sausages can be from pH 4 to 7, or from  $a_w$  <0.6 to > 0.95 [24,25]. A study [26] on reducing the  $a_w$  in local sausage production processing has been published.

#### 3.1. *Changing the pH of fermented sausages during ripening*

The mean pH of all groups of fermented sausages during the ripening significantly declined and at the end of maturation was significantly lower in the wider diameter sausages, and in sausages inoculated with starter culture. On day 0, the mean pH of the sausage stuffing was  $6.14 \pm 0.10$ . During ripening, the mean pH of smaller diameter sausage declined, so that at the end of the ripening period (after 18 days) it was  $5.32 \pm 0.03$ , and for sausages of the same diameter with the addition of starter culture, the pH was  $5.03 \pm 0.02$ . This large difference between the average pHs was statistically significant ( $p < 0.05$ ). Sausages with wider diameter also showed pH declines after ripening, so in these sausages without and with the addition of starter culture, the mean pH was  $5.09 \pm 0.07$ , and  $4.91 \pm 0.04$ , respectively (a statistically significant difference,  $p < 0.01$ ). At the end of maturation (day 35), the mean pH of smaller diameter sausages with no added starter culture, was  $5.40 \pm 0.06$ , and the sausages with added starter culture, had a mean pH of  $5.24 \pm 0.08$  (a statistically significant difference,  $p < 0.05$ ). At the end of maturation, wider diameter sausages without and with addition of starter culture, were mean pH  $5.24 \pm 0.03$ , and  $5.07 \pm 0.05$ , respectively (a statistically significant difference,  $p < 0.05$ ).

#### 3.2. *Changing the $a_w$ values in fermented sausages during ripening*

During the ripening process, both fermented sausages with narrower and wider diameter showed significantly decreased  $a_w$ , but smaller  $a_w$  decreases occurred in sausages with the starter cultures added.

The mean  $a_w$  for sausage stuffing on day 0 was  $0.9695 \pm 0.0007$ . The  $a_w$  declined during the ripening period, and at the end, on day 18,  $a_w$  in smaller diameter sausages with no added starter culture was  $0.9110 \pm 0.0006$ , and this was statistically significantly lower than in sausages of the same diameter with the addition of starter culture ( $p < 0.01$ ;  $a_w$   $0.9211 \pm 0.0005$ ). On day 18, the mean  $a_w$  of wider diameter sausages with no starter was  $0.9346 \pm 0.0008$ , and this was significantly lower ( $p < 0.05$ ) than the mean  $a_w$  of wider diameter sausage with starter ( $0.9361 \pm 0.0008$ ). At the end of maturation, the mean  $a_w$  of smaller diameter sausages was  $0.9200 \pm 0.0007$ , and the mean  $a_w$  of wider diameter sausages was  $0.9235 \pm 0.0004$ .

#### 4. Conclusion

The proper, controlled fermentation of raw sausages produces largely safe products that do not contain foodborne pathogens which could cause disease, among which is *Y. enterocolitica*. In fermented sausages, during ripening and drying, different parameters are used to stop/slow/inhibit the growth of pathogenic bacteria, and these parameters act simultaneously. Known parameters include pH decrease, presence of lactic acid, decreases in  $a_w$ , inhibitory effect of smoke (if the sausages are smoked), presence of starter culture and action of metabolites (bacteriocins), etc.

Inactivation of pathogenic bacteria during the ripening of sausages involves control of their growth, and it is a key step in the production of fermented sausages. Reduction of their number ends or is insufficient if the ripening process does not last long enough or is not optimal (incorrect starter culture, temperature, humidity, circulation), which is why this process should be controlled.

In many European countries, demand for traditional food products is increasing. These products are foods with strong regional characteristics and of local origin, which should be protected and promoted, as a characteristic form of local traditional food production.

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# Antimicrobial effect of oregano-chitosan double coatings on *Listeria monocytogenes* in meat products

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**Abstract.** The aim of this study was to investigate the effect of oregano and chitosan applied as edible coatings on the chemical and microbiological properties of different type of meat products. The main focus from the microbiological aspect was on *Listeria monocytogenes* as a pathogen that can survive for a long period on this type of product and, hence, present a risk for consumers. *In vitro* testing showed chemical quality after 7 and 14 days' storage of the three types of products was stable. Promising results were obtained for the microbial analysis, whereby large reductions in *L. monocytogenes* numbers after 7 and 14 days' storage at 4°C was measured in the coated products. The results indicated the chitosan and oregano combination can be an effective inhibitor of *L. monocytogenes* growth in chilled meat products.

## 1. Introduction

The food industry is focusing on finding modern methods and technologies to increase the shelf life of foods. The susceptibility of ready-to-eat meat products to spoilage is an economic problem for producers, and various methods have been applied to enhance the shelf life of these foods [1]. On the other hand, consumer demands for healthy meals that are free of chemical preservatives are greater now than in the past.

Biodegradable, edible chitosan films combined with plant extracts as double coatings have recently been widely used to enhance the qualitative and microbial stability of food [2], [3], [4]. Edible coatings are applied on the surface of the food in thin mono- or multi-layers. Direct incorporation of essential oils in foods such as meat products will result in the immediate reduction of the bacterial population but can alter the sensory characteristics of added food. This incorporation of essential oils in edible films may be particularly interesting, and some studies showed that oregano and garlic oil were, for example, effective in whey protein-based films against *Staphylococcus aureus*, *Salmonella* Enteritidis, *Listeria monocytogenes*, *Escherichia coli* and *Lactobacillus plantarum* [5].

This study was performed using oregano-chitosan double coating to improve the microbial stability of meat products against *L. monocytogenes*. The control and coated meat samples were stored in plastic containers at 4°C for two weeks. The quality was evaluated by determining the water and fat content and the microbial safety by following the numbers of *L. monocytogenes* as indicators of the potential antimicrobial effect of the added extracts as coatings.

## 2. Materials and methods

### 2.1. Materials

Chitosan was purchased from Sigma Aldrich (St. Louis, USA), while glycerol, Tween 80 and acetic acid were from Merck, Germany. All chemicals were of analytical grade. The alcohol oregano extract was obtained with supercritical extraction with CO<sub>2</sub> from dried oregano herb.



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## 2.2. Preparation of the edible coatings

Chitosan solution (1%, w/v) was prepared by dissolving 1 g of chitosan in 100 mL of acetic acid (1% v/v) with continuous stirring at room temperature ( $20\pm 2^\circ\text{C}$ ). After the complete dissolution of the chitosan, 0.2 g of glycerol and 0.2 g of Tween 80, were added and the solution was stirred for another 30 min.

The oregano extract was prepared by dissolving 1g of extract in 10 ml 97% ethanol.

## 2.3. Preparation of the meat samples

Meat products (smoked pork neck, ham and smoked beef tenderloin), commercially produced, were purchased at retail. The plastic packaging was removed and slices were cut. The double coating was applied by brushing the slices on their surface with the oregano extract and letting it dry for 20 min. Then, the second coating, chitosan, was applied, again by brushing and then drying for additional 20 min.

After the drying of the two coatings, *L. monocytogenes* ATCC 13932 was added to the meat products. The bacterial concentration was approximately  $1.5 \times 10^6$  cfu/ml and the coated slices were dipped for 30 seconds in the bacterial suspension. Four groups of meat product slices were prepared.

1. Meat products for chemical and sensory analysis without chitosan and oregano, no contamination with *L. monocytogenes*; A – smoked pork neck, B – ham, C – smoked beef tenderloin.
2. Meat products for chemical and sensory analysis with chitosan and oregano, no contamination with *L. monocytogenes*; CA – smoked pork neck, CB – ham, CC – smoked beef tenderloin.
3. Meat products for microbiological analysis without chitosan and oregano and contaminated with *L. monocytogenes*; AM – smoked pork neck, BM – ham, CM – smoked beef tenderloin.
4. Meat products for microbiological analysis with chitosan and oregano and contaminated with *L. monocytogenes*, CAM – smoked pork neck, CBM – ham, CCM – smoked beef tenderloin.

All of the prepared meat product slices were stored at  $4^\circ\text{C}$  and were analysed on days 7 and 14 of storage.

## 2.4. Quality control of meat samples

Water and moisture contents of the coated and uncoated meat slices were analysed using reference methods. For the total fat content, analysis was according to ISO1443:1973 and moisture content was according to ISO 1442:1997.

## 2.5. Microbiological analysis of meat samples

Detection and enumeration of *L. monocytogenes* was performed according to ISO 11290:2017. For the isolation of *L. monocytogenes*, approximately 25 g of each sample was homogenised with 225 mL of half-strength Fraser broth in a stomacher for 2 minutes. This homogenate was then incubated at  $30^\circ\text{C}$  for 24 h. An aliquot of 1 mL was transferred to tubes containing Fraser broth supplemented with Fraser selective supplement and incubated at  $37^\circ\text{C}$  for 48 h. The cultures were streaked onto plates containing the *Listeria* agar Ottaviani & Agosti and incubated at  $37^\circ\text{C}$  for 24 h. Afterwards, 3-5 suspect colonies were selected for confirmation. The confirmation of *L. monocytogenes* colonies after isolation on suitable media was based on several methods, including Gram staining and measurement of haemolytic activity on sheep blood agar, the carbohydrate utilisation pattern, the catalase reaction and tumbling motility.

## 2.6 Sensory analysis of the meat products

A six member trained panel evaluated the meat product slices according to standard protocol (white light illumination, room temperature of  $23\pm 2^\circ\text{C}$ , relative humidity of 50%). Ten gram samples were coded randomly and were served in white plastic plates along with a glass of water for neutralisation

of the taste. The sensory panel evaluated the attributes of colour, odour and general appearance using a 9 point intensity scale [6].

### 2.7 Statistical analysis

All data were analysed statistically ( $n=6$  and  $p=0.05$  or 5% significance level) by one way analysis of variance homogeneity test and Duncan's Multiple Range Test (DMRT) using SPSS-16.0 software package for standard methods [7].

## 3. Results and discussion

### 3.1 Chemical analysis

The chemical parameters, water and fat content, are given in Table 1. These parameters are the most variable during storage, so were chosen as indicators of the products' stability. The main characteristic of the edible coatings was anticipated to be their antimicrobial effect against *L. monocytogenes*. However, chemical analyses proved the double coating also exhibited good barrier properties, since there was no significant change in the water and fat content of the coated meat slices, meaning the coatings prevented the meat products (CA, CB and CC) from drying which could later cause auto-oxidation of lipids and loss of sensorial attributes. The uncoated meat products (A, B and C) showed loss of water during storage, proving they were more susceptible to drying than their coated counterparts.

**Table 1.** Chemical analysis of the meat product slices

Meat product group	Water content (%)		Fat content (%)	
	7 day	14 day	7 day	14 day
CA	66.5±1.5 <sup>a</sup>	65.1±2.1 <sup>a</sup>	2.5±0.5 <sup>a</sup>	2.9±0.4 <sup>a</sup>
A	68.5±2.0 <sup>a</sup>	65.4±0.9 <sup>b</sup>	3.0±0.7 <sup>a</sup>	3.2±0.2 <sup>a</sup>
CB	71.6±1.9 <sup>a</sup>	72.3±1.4 <sup>a</sup>	2.4±0.4 <sup>a</sup>	2.6±0.3 <sup>a</sup>
B	73.2±2.2 <sup>a</sup>	70.0±0.6 <sup>b</sup>	2.5±0.3 <sup>a</sup>	2.4±0.1 <sup>a</sup>
CC	53.1±1.8 <sup>a</sup>	58.2±2.9 <sup>a</sup>	2.5±0.3 <sup>a</sup>	2.4±0.2 <sup>a</sup>
C	60.4±2.8 <sup>a</sup>	54.5±2.8 <sup>b</sup>	2.6±0.6 <sup>a</sup>	2.9±0.3 <sup>a</sup>

Mean values and standard deviation, ( $n = 3$ ); Different small letters within a row for each analysis indicate significant differences due to storage time within the same sample; Meat product slices with double coating, CA – smoked pork neck, CB – ham, CC – smoked beef tenderloin; meat product slices without coating, A – smoked pork neck, B – ham, C – smoked beef tenderloin.

### 3.2 Microbiological analysis

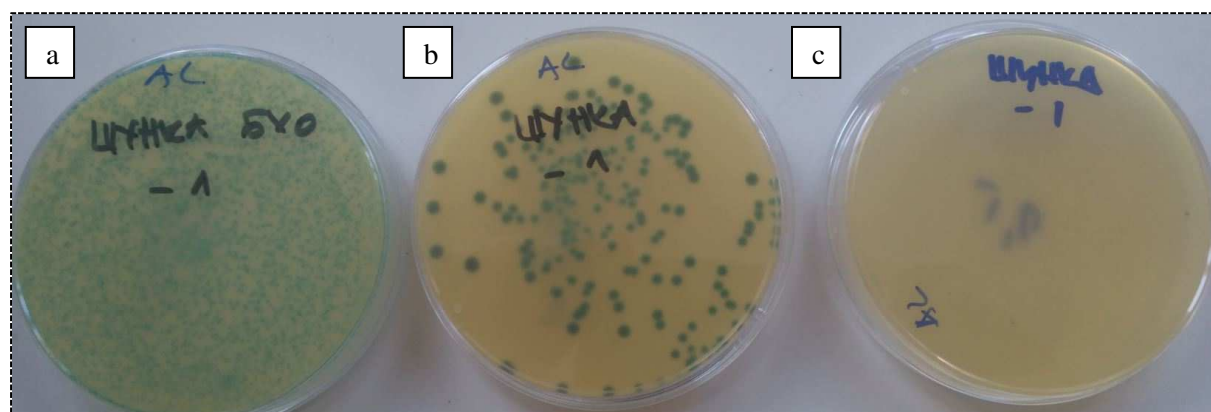
Our investigation showed that *L. monocytogenes* remained viable on the meat products for the examined 14-day period (Table 2). That will surely influence the expected shelf-life of such products, and if they do contain this pathogen, could affect consumers' health.

Contact of *L. monocytogenes* with chitosan-oregano double coating resulted in decreased bacterial populations (Table 2 and Figures 1-3). Populations of *L. monocytogenes* ATCC 13932 inoculated on the meat products in the presence of chitosan-oregano double coating decreased from 6 log<sub>10</sub> cfu/g to undetectable levels after 14 days at 4°C in two types of meat products (smoked pork neck and smoked beef tenderloin) while in the third meat product group (ham), a large decrease in the microbial load was measured. The mechanism of this antimicrobial activity can be explained in various ways. Some authors have suggested that the interaction between positively-charged chitosan molecules and negatively-charged microbial surfaces results in the disruption of cell membranes, leakage of intracellular constituents, and ultimately, microbial cell death [8]. Concerning the meat products without coating, microbiological analysis showed stable populations of the inoculated pathogen.

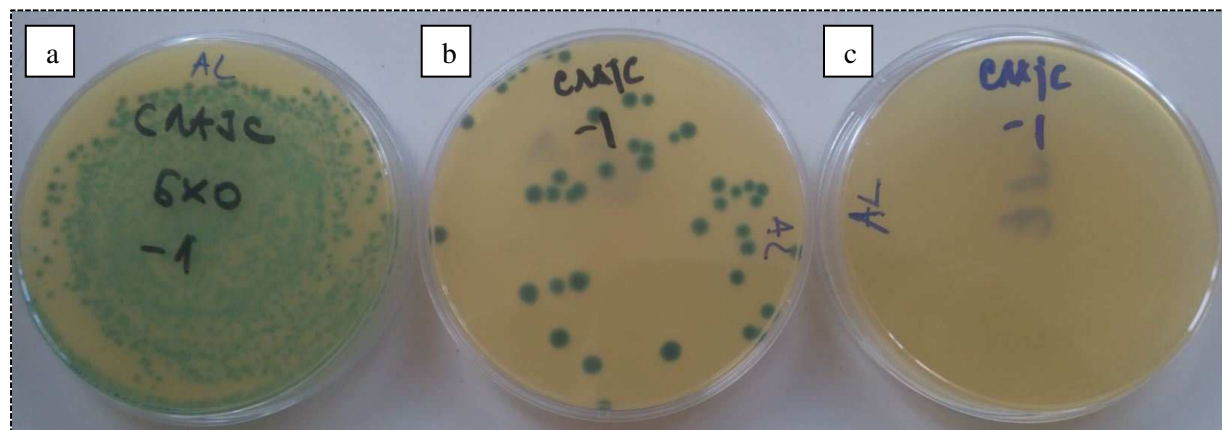
**Table 2.** *L. monocytogenes* numbers on sliced meat products with and without coating during 14 days' storage at 4°C

Meat product group	<i>Listeria monocytogenes</i> log cfu/g	
	7 days	14 days
CAM	3.36	0
AM	5.32	4.70
CBM	3.83	1.47
BM	5.66	5.08
CCM	2.96	0
CM	5.53	4.23

Meat product slices with coating, CAM – smoked pork neck, CBM – ham, CCM – smoked beef tenderloin; meat product slices without coating, AM – smoked pork neck, BM – ham, CM – smoked beef tenderloin.

**Figure 1.** Presumptive *Listeria* derived during storage from AM – smoked pork neck, no coating, and; CAM – smoked pork neck with coating. AM after 7 days, b) CAM after 7 days and c) CAM after 14 days**Figure 2.** Presumptive *Listeria* derived during storage from BM – ham no coating, and; CBM – ham with coating. a) BM after 7 days, b) CBM after 7 days and c) CBM after 14 days





**Figure 3.** Presumptive *Listeria* derived during storage from CM – smoked beef tenderloin, no coating, and; CCM – smoked beef tenderloin with coating. a) CM after 7 days, b) CCM after 7 days and c) CCM after 14 days

### 3.3 Sensory analysis

The trained panellists evaluated the meat products during storage in terms of colour, odour and general appearance (Table 3). As expected, the coated meat product slices had slightly lower scores compared to their corresponding controls, but were still very well graded, with more than 75% of panellists finding them acceptable. At the beginning, the acceptability difference was the most noticeable, being around 12% difference between control and coated meat products, and was most noticeable for ham. After the first week, the score difference was 9-10% between control and coated meat products, while after the second week of storage, this sensory difference was even smaller and ranged around 6-7%. These results confirm that the edible coating, besides its beneficial effect on the products' stability, also, had good sensorial characteristics, since oregano is among the most common herbs, and consumers usually accept its flavour without problem.

**Table 3.** Sensory analysis of the coated and uncoated meat slices

Meat product group	Colour		Odour		General appearance	
	7 days	14 days	7 days	14 days	7 days	14 days
CA	7.4 ± 0.42 <sup>a</sup>	7.0 ± 0.52 <sup>b</sup>	8.3 ± 0.82 <sup>a</sup>	7.6 ± 0.56 <sup>b</sup>	8.0 ± 0.67 <sup>a</sup>	7.6 ± 0.51 <sup>b</sup>
A	8.1 ± 0.31 <sup>a</sup>	7.5 ± 0.52 <sup>b</sup>	8.5 ± 0.84 <sup>a</sup>	7.9 ± 0.69 <sup>b</sup>	8.4 ± 0.51 <sup>a</sup>	7.9 ± 0.42 <sup>b</sup>
CB	8.0 ± 0.70 <sup>a</sup>	7.7 ± 0.48 <sup>b</sup>	8.4 ± 0.51 <sup>a</sup>	7.6 ± 0.69 <sup>b</sup>	8.0 ± 0.47 <sup>a</sup>	7.9 ± 0.31 <sup>b</sup>
B	8.7 ± 0.67 <sup>a</sup>	8.6 ± 0.69 <sup>b</sup>	8.4 ± 0.84 <sup>a</sup>	7.9 ± 0.31 <sup>b</sup>	8.6 ± 0.51 <sup>a</sup>	8.3 ± 0.48 <sup>b</sup>
CC	7.9 ± 0.52 <sup>a</sup>	7.3 ± 0.82 <sup>b</sup>	8.2 ± 0.42 <sup>a</sup>	7.6 ± 0.51 <sup>b</sup>	8.5 ± 0.70 <sup>a</sup>	8.0 ± 0.47 <sup>b</sup>
C	8.3 ± 0.31 <sup>a</sup>	8.0 ± 0.48 <sup>b</sup>	8.5 ± 0.84 <sup>a</sup>	8.3 ± 0.67 <sup>b</sup>	8.8 ± 0.42 <sup>a</sup>	8.6 ± 0.51 <sup>b</sup>

Mean values and standard deviation, ( $n = 6$ ); Different small letters within a row indicate significant differences due to storage time within the same sample.

Meat product slices with double coating, CA – smoked pork neck, CB – ham, CC – smoked beef tenderloin; meat product slices without coating, A – smoked pork neck, B – ham, C – smoked beef tenderloin.

#### 4. Conclusion

Oregano-chitosan double coatings can improve the microbial safety of meat products during refrigerated storage and increase the shelf life while maintaining the quality parameters. However, there is still a requirement to improve and standardise the coating procedures according to food industry needs (reduced costs and increased shelf life) and to meet consumer demands without altering the sensory characteristics of the meat products.

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# Examination of meat preparations in order to control process hygiene in retail

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**Abstract.** The production and trade of meat preparations (minced meat and semi-finished meat products, including fresh sausages) are registering significant annual increases in Serbia. There is an increasing number of specialized plants, as well as suppliers who directly supply consumers with this type of meat preparation. The aim of this paper is to determine the microbiological risks in the meat preparations production process by taking samples from retail facilities in order to verify HACCP compliance. HACCP systems and good hygiene practices as their pre-requisite programs, require food business operators to identify potential hazards that threaten product safety in order to eliminate or control them. Over 27 months, 297 samples of meat preparations were taken from nine retail stores. *Escherichia coli* was detected in 5% (16/297) of the meat preparations, and *Salmonella* spp. were found in 1.6% (5/297). The results obtained are signals for initiating corrective measures in the production processes and improving current sanitary procedures.

## 1. Introduction

Meat and meat products are recognised as good sources of high biological value proteins, liposoluble vitamins, minerals and bioactive compounds. Changes in socioeconomic factors in recent years have increased the consumer's willingness for ready-to-eat (RTE) food [1]. Baltić believes the fast food and all related spreading phenomena are related to the quick and efficient service of this type of food to the consumers. The true symbol of Serbian national cuisine, above all is grilled meat [1].

Meat preparations are obtained from fresh meat which has been minced and to which other foods, spices and additives, are added, but without modifying muscle structure and its fibres in order to eliminate the fresh meat characteristics. These meat preparations are intended for use after heat treatment [2].

However, the meat as raw material can be microbiologically burdened by inadequate technological operations combined with a lack of appropriate hygiene measures at the slaughterhouse level. Also, during storage and transport, an additional microbiological burden can be added. Therefore, preventive hygiene measures must be taken to obtain a hygienically correct product.

The retail meat trade is on a small scale, where customer is served by the vendor [3]. Hygienic sanitary conditions in retail are more demanding than in wholesale [4]. Most foodborne diseases occur due to inadequate handling with the result of food contamination, or due to previously delivered contaminated food [5]. Preventive hygienic measures in production, processing and trade are aimed at ensuring product safety and quality of meat preparations. The hazard analysis and identification of critical control points (HACCP) is a legal obligation for food business operators, as it is the most effective system for reducing or eliminating contamination during food production. For successful implementation of the HACCP system, it is necessary to obtain data on microbiological risks. Food business operators must have self-control plans where microbiological testing is defined (type of examined microorganisms, dynamics, limits, etc.) in order to validate HACCP system. Meat



preparations must have a microbiota that fulfils set criteria, so there is a need for greater hygienic control, since number and type of microorganisms present depends on hygienic performance. Also, meat preparations are most often sold unpacked, and therefore, the risk of microbiological contamination is higher.

Hygienic measures in production, processing and trade are aimed at ensuring product safety and preventing the rapid deterioration and quality of meat. HACCP is the most effective system for reducing or eliminating contamination during food production. For successful implementation of the HACCP system, it is necessary to have data on microbiological contamination. For the validation and verification of the HACCP system, a food business operator is required to have continuous microbiological data that follows specific dynamics defined in the self-control plan that the operator itself prescribes in accordance with legislations. Microbiological data on hygienic indicators are important for assessing the level of hygiene in the facilities in which semi-products are produced, and the categorization of the risk is assessed by monitoring indicator microorganisms.

By following the presence of indicator microorganisms, it is possible to indirectly monitor the likely presence of pathogenic microorganisms such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Escherichia coli* and *Yersinia enterocolitica*. These food pathogens are considered to be major sources of human infections [6]. In order to prevent contamination, during processing and trafficking, it is very important to apply hygienic procedures on work surfaces that come into direct contact with food, tools, equipment and workers hands [7]. A high level of hygiene in the work environment is fundamental for preventing microbiological contamination [8]. Inadequate cleaning and disinfection can lead to microbial contamination and reduction of product shelf life [9]. Workers who come in direct contact with food must be trained and informed about the importance of proper food handling. Their hygiene and work habits must be properly applied. Constant attention and compliance with the good hygienic practice (GHP) within HACCP is required. Aksoydan states that apart from legal requirements, hygiene and adequate training of workers, it is a key factor in food safety [10]. Wearing gloves, both in the preparation of raw materials for the production of meat preparations, and in manipulating the finished product, is considered an effective way of preventing the transmission of bacteria to food [11]. According to Tomasevic, 93.5% of slaughterhouses, meat processing and trade chains in Serbia have an implemented and certified HACCP system [12]. Despite the legal requirements for the implementation of GHP and HACCP, cross contamination remains an important factor in food poisoning and epidemics that occurred in fast food facilities [13]. The application of standard sanitary operative procedures (SSOP) is necessary in order to carry out proper sanitation. Improper sanitation is directly related to various cases of foodborne disease epidemics. Several pathogenic microorganisms, including *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella* spp. and enteropathogenic strains of *Escherichia coli* can survive on different surfaces over a period of several hours to a few days, and form a biofilm [14].

*E. coli* is a faecal contamination indicator and an indicator of compliance with the basic principles of GHP during the production process [15]. *Salmonella* spp. was monitored as a safety criterion suitable for application in retail. It is recommended that sampling occurs at the last moment when the final product is under the direct control of the food business operator that produced it, in order to carry out risk analysis. Microbiological criteria are stated in Serbian legislation [16], as well as corrective measures in case of unsatisfactory results.

## 2. Materials and Methods

In order to assess the microbiological risk involved in the production of meat preparations (formed minced meat – kebabs and burgers, fresh sausages), samples were taken in accordance with the self-control plans of the businesses that participated in the study. The frequency of sampling is defined by current legislation, or by the food business operator itself with the consent of the official veterinary inspection service. In a period of 27 months (October 2016 to the end of 2018), 297 samples of formed minced meat (kebabs, burgers or fresh sausages) were taken from 9 retail stores. In the first 6 months,

samples were taken from each store twice a month, and further samples taken once a month. Each sample consisted of 5 units. Samples were tested for the presence of *E. coli* and *Salmonella* spp.

The limits for *E. coli* (Table 1) are 500 cfu/g to 5000 cfu/g as the two limit values (m and M), respectively, so a three-class plan is applied. The results are interpreted in three categories: as satisfactory (if all obtained values are less than 500 cfu/g or  $< m$ ), as acceptable (if a maximum 2 (c) of 5 (n) obtained values are between 500 and 5000 cfu/g (between m and M), and the other obtained values are less than or equal to 500 cfu/g ( $\leq m$ )), or as unsatisfactory (if one or more of the obtained values are greater than 5000 cfu/g ( $> M$ ), or if more than 2 (c) of the tested 5 (n) units have values between 500 and 5000 cfu/g (between m and M). This type of interpretation is commonly used for hygiene criteria in the production process [17].

The limits for *Salmonella* spp. are absence in 10 g of any tested unit in the sample of 5, as a one-limit (M), two class plan is applied. The results obtained are interpreted as satisfactory (if *Salmonella* is not found in any tested unit) or as unsatisfactory (if the presence of *Salmonella* spp. is detected in any of the sample units). This type of interpretation is commonly used for food safety criteria in the production process [17].

**Table 1.** Limits for the numbers of *E. coli* in meat preparations

Food Category	Microorganisms	Sampling plan		Limits		Analytical reference method	Phase where the criterion applied	Action in case of unsatisfactory results
		n	C	m	M			
Meat preparation	<i>E. coli</i>	5	2	500 cfu/g	5000 cfu/g	EN ISO 16649-2	At the end of the production process	Improvement of process production hygiene and improving the choice and origin of raw material

The following methods were used for laboratory testing of the meat preparation samples: SRPS ISO 16649-2: 2008 Microbiology of food and animal feeding stuffs - Horizontal method for determining the number of s-glucuronidase positive *Escherichia coli* - Part 2: Colony count technique at 44 ° C using 5-bromo-4-chloro-3-indolyl sD- glucuronide [18].

SRPS EN ISO 6579-1: 2017 Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp. [19].

### 3. Results and discussion

In a period of 27 months, 297 meat preparations (kebabs, burgers or sausages) from 9 retailers were examined. Of the 108 tested meat preparations collected in the first 6 months (Table 2), the presence of *E. coli* was found in 11 meat preparations, which is 10.2% (11 out of 108), while *Salmonella* spp. was determined in 4 meat preparations, which is 3.7% (4 out of 108).

**Table 2.** Presence of *Escherichia coli* and *Salmonella* spp. in meat preparations during the first 6 months of the study

Microorganisms	Number of samples	Positive samples	%
<i>Escherichia coli</i>	108	11	10.2

*Salmonella* spp.

108

4

3.7

In the next 21month period (Table 3), 189 meat preparations were examined. The presence of *E. coli* was found in 5 meat preparations, which is 2.6% (5 of 189). *Salmonella* spp. was determined in one meat preparation, which is 0.5% (1 of 189).

**Table 3.** Presence of *Escherichia coli* and *Salmonella* spp. in meat preparations during the last 21 months of the study

Microorganisms	Number of samples	Positive results	%
<i>Escherichia coli</i>	189	5	2.6
<i>Salmonella</i> spp.	189	1	0.5

The results obtained in this study are worse than those obtained in Ireland, where the prevalence of *Salmonella* spp. was from 0.1%, and *E. coli* 0.2% in similar products [20]. Regarding data from 2010, published by EFSA and ECDC and originating in 12 countries in the EU, 2.8% of such meat preparations contained *Salmonella* spp., and 0.6% contained *E. coli* [21].

Based on the microbiological criteria and given limit values (m and M), i.e., when a three-class plan was applied, the results obtained by examining 297 samples were interpreted as satisfactory, acceptable or unsatisfactory, or based on a two-class plan, the results were interpreted as satisfactory or unsatisfactory (Table 4). Among these 297 meat preparations examined, *E. coli* was not detected in 94.6% of the meat preparations, while *Salmonella* spp. were not detected in 98.3% of the meat preparations. We speculate that such results are likely to have benefitted from continuous and intense education of employees plus positive and good management.

**Table 4.** Interpretation of microbiological results for meat preparations at retail over the entire examined period

Microorganisms	Number of samples	Satisfactory Results	Acceptable results	Unsatisfactory results
<i>Escherichia coli</i>	297	281	9	7
<i>Salmonella</i> spp.	297	292	/	5

#### 4. Conclusion

A popular practice in the meat preparation trade at retail is the cooking and sale of unpacked product, which causes additional microbiological risk over that of packaged produce. When unsatisfactory results for the hygienic indicator *E. coli* occur, as they did in 2.4% of the samples of meat preparations we examined, the food business operators concerned must take appropriate corrective measures. Corrective measures should be determined in each operator's self-control plan, and focus on improving process hygiene. Additionally, selection of more hygienic raw material is needed. Corrective actions should include: what needs to be done with the production batch with unsatisfactory results; who should be notified of the nonconforming production lot; how to determine the cause of non-compliance, and; how to eliminate the cause of non-compliance so it does not recur. Hygiene non-compliance demands corrective measures be implemented (increased sampling frequency, increased cleaning and disinfection, change of disinfection products, improved selection of raw material, monitoring the mode of transport, maintenance of cold chain, improved personal hygiene of workers, tools and equipment, revision of the HACCP plan). One of the preventive measures for food safety management is the use of good quality equipment that facilitates effective cleaning and sanitation. Production facilities must have implemented HACCP plans, and so continuous and intensive

education of employees must be conducted. Before meat preparations are exposed to thermal treatment (i.e., before they are cooked by the retailer), only properly implemented sanitary procedures help to reduce the presence of microorganisms, some of which may be potentially pathogenic.

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## Collaborative effect of fat reduction and $\alpha$ -tocopherol incorporation on oxidative stability in beef sausages

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**Abstract.** This study focuses on the changes in oxidative stability and sensory properties of reduced fat and/or  $\alpha$ -tocopherol incorporated sausages during storage at 4°C for 3 months. In order to examine these changes, sausages were formulated with 20% fat and 20% fat+200 ppm  $\alpha$ -tocopherol, coded as C20A0 and C20A1, respectively. Sausages formulated with 10% fat (C10A0) and 10% fat+200 ppm  $\alpha$ -tocopherol (C10A0) were low fat sausages. Reduction of fat by 50% or adding  $\alpha$ -tocopherol initially increased the peroxide values of sausages, but at the end of storage, conversely, reduction of fat and  $\alpha$ -tocopherol addition retarded lipid peroxidation as well as malonaldehyde generation ( $p < 0.05$ ). The highest thiobarbituric acid reactive substance values were recorded for C20A0 sausages initially and at the end of the storage ( $p < 0.05$ ). Even though thiobarbituric acid reactive substance values of sausages with 20% fat were higher, the initial rancid taste of all sausages were similar at month 0, but differences in the rancid taste of sausages began to be revealed with increasing storage time ( $p < 0.05$ ). No significant differences were found in the general acceptability of all the sausages during the 3-month storage period ( $p > 0.05$ ).

### 1. Introduction

Meat and meat products are valuable sources of important food components such as proteins, essential amino acids, minerals and vitamins [1]. Despite the nutritious value of meat and meat products [2], fat contents of meat products have caused negative perceptions in consumers towards consumption of these products due to the fat-linked health problems such as cardiovascular diseases, obesity and some cancer types [3]. The World Health Organization also recommended that total fat consumption should not exceed 30% of total energy intake [4,5]. However, emulsion type meat products can be formulated with fat content up to 30 % [6].

Lipid oxidation reactions that limit the shelf life of meat products are induced from high fat content. These reactions create unfavorable changes in product quality besides their adverse effect on health due to generation of toxic compounds as a result of chain reactions [7]. Oxidative changes can be retarded by lowering the fat content or with antioxidant compounds. Sequestration of free radicals from the medium, chelation of metallic ions, inhibition of free radical-producing enzymes, activation of endogenous antioxidant enzymes, prevention of lipid peroxidation, prevention of DNA damage,





prevention of protein modification and sugar destruction are some important mechanisms of antioxidants [8].

Given these circumstances, the need for low fat or antioxidant-added meat products has arisen [9]. Nevertheless, when the fat content is decreased, acceptance of meat products by consumers weakened due to the decreased mouthfeel, texture and juiciness properties provided from fat. Thus, designing low fat meat products is a reasonably troublesome topic [10,11].

In this study, the effects of decreasing beef fat content from 20% to 10% in sausages and incorporating  $\alpha$ -tocopherol in the sausage formulation on oxidative changes and some sensory properties during 3-month storage at 4°C were studied.

## 2. Materials and methods

Four different emulsion type sausage formulations were prepared (Table 1). Minced beef and beef fat were purchased from a local butcher,  $\alpha$ -tocopherol was obtained from Kimbiatek (İstanbul-Turkey). Minced beef, curing ingredients and half of the ice were homogenized and ground for 1 min in a cutter (Alpina-Schweiz). Other ingredients added (to 5000 g of sausage batter) to all sausages were: 1.5% salt, 0.15% sugar, 0.15% sodium tripolyphosphate, 0.09% ascorbic acid, 150 ppm sodium nitrite, 3% powdered milk, 3% sodium caseinate, 4% starch, 1.5% bread crumbs, 0.9% spices. Fat,  $\alpha$ -tocopherol (depending on formulation; Table 1), the other ingredients and the remainder of the ice was added to the meat and mixed for one more minute. Finally, batters were homogenized for 3 minutes. Sausage batters were stuffed into casings and smoked in smokehouse at 40°C for 2 h (Afos-England), then heat treated in a boiling vessel until the core temperature reached 70°C. Once heating was completed, sausages were cooled, vacuum packaged and stored at 4°C for 3 months. Oxidative changes of sausages were determined in terms of peroxide values [12] and thiobarbituric acid reactive substances (TBARS) [13]. Sensory properties of sausages were evaluated in terms of oiliness, rancid taste and general acceptability (1: not like, 9: extremely like). Data was analyzed by ANOVA and Duncan's Post-Hoc tests using SPSS 23 software.

**Table 1.** Formulation of sausages

Sausage*	Beef meat (g)	Beef fat (g)	Ice (g)	$\alpha$ -Tocopherol
C20A0	3000	1000	1000	-
C20A1	3000	1000	1000	200 ppm
C10A0	3000	500	1500	-
C10A1	3000	500	1500	200 ppm

\*Sausage denomination: C20A0 (Control group, 20% beef fat), C20A1 (Control group, 20% beef fat with 200 ppm  $\alpha$ -tocopherol), C10A0 (Reduced fat control group, 10% beef fat), C10A1 (Reduced fat control group, 10% beef fat with 200 ppm  $\alpha$ -tocopherol).

## 3. Results and discussion

Peroxide values of sausages were between 0.391 and 2.749 meqO<sub>2</sub>/kg during the 3-month storage (Table 2). At the beginning, the lowest and highest peroxide values were measured in C20A0 and C10A1 sausages, respectively ( $p < 0.05$ ). Storage period had a significant effect on the sausages' peroxide values ( $p < 0.05$ ). During storage, peroxide values of C20A0 sausages increased continuously up to month 3 ( $p < 0.05$ ). Peroxide values of C20A1 sausages increased after the 1st month of storage, but in C10A0 sausages, values decreased after 2 months of storage ( $p < 0.05$ ). At the end of the storage, lipid peroxidation was affected by fat reduction and  $\alpha$ -tocopherol addition ( $p < 0.05$ ). Beef fat at the higher level (20%) induced higher peroxide values than were measured in 10% beef fat sausages ( $p < 0.05$ ). Addition of  $\alpha$ -tocopherol did not retard the oxidation of sausages formulated with 10% beef fat ( $p < 0.05$ ). Incorporating  $\alpha$ -tocopherol to pork patties delayed the lipid oxidation after 20 days of

storage [14]. In our study, the peroxide values of all sausages were lower than 25 meqO<sub>2</sub>/kg, which is the limit for fatty foods [15].

**Table 2.** Peroxide values of sausages (meqO<sub>2</sub>/kg)

Sausage	Month 0	Month 1	Month 2	Month 3
C20A0	0.777±0.018 <sup>b,z</sup>	1.107±0.097 <sup>ab,yz</sup>	1.735±0.344 <sup>ab,y</sup>	2.749±0.743 <sup>a,x</sup>
C20A1	1.020±0.099 <sup>ab,y</sup>	0.720±0.110 <sup>bc,y</sup>	2.229±0.577 <sup>a,x</sup>	1.688±0.114 <sup>b,x</sup>
C10A0	1.058±0.111 <sup>ab,x</sup>	1.259±0.560 <sup>a,x</sup>	1.193±0.203 <sup>c,x</sup>	0.524±0.226 <sup>c,y</sup>
C10A1	1.717±0.697 <sup>a,x</sup>	0.393±0.001 <sup>c,y</sup>	1.252±0.114 <sup>c,x</sup>	0.391±0.004 <sup>c,y</sup>

Data are presented as the mean values of replications ± standard deviation. abc: Means with the different letter in the same column are significantly different (p<0.05); Data are presented as the mean values of replications ± standard deviation. xyz: Means with the different letter in the same row are significantly different (p<0.05).

TBARS values of sausages are given in Table 3. The highest initial TBARS value was determined in the control group produced with 20% beef fat without  $\alpha$ -tocopherol (p<0.05). All sausage groups except C20A0 showed similar initial TBARS values. Using antioxidant had an effect on TBARS value only in the presence of 20% fat in the formulation. Reduction of fat in the formulation lowered the TBARS values both initially and at the end of the storage (p<0.05). During storage, TBARS values of C20A1 sausages increased up to the 2nd month, but afterwards, decreased TBARS values were measured, even though the final values were higher than initial values (p<0.05). TBARS values of C10A0 and C10A1 sausages fluctuated during the 3-month storage. At the end of the storage, sausages formulated with 20% beef fat showed higher TBARS values than sausages formulated with 10% beef fat (p<0.05). Similar to the initial pattern of peroxide values, using  $\alpha$ -tocopherol was found to effectively retard the oxidative changes only in sausages with 20% fat. The TBARS values of all sausages remained within the acceptable limit of TBARS, i.e. lower than 2.0 mg MA/kg sample [13] throughout the 3-month storage, most probably because of vacuum packaging.  $\alpha$ -tocopherol showed its antioxidant effect by singlet oxygen scavenging and radical chain reaction breaking [16]. C10A0 and C10A1 sausages showed no differences from each other while these values were lower than other two sausage types. Similar to our results, Olivares et al. [6] reported that dry fermented sausages formulated with 30% fat had higher TBARS values than samples with 10% fat content. In another study, using  $\alpha$ -tocopherol in pork patties retarded the oxidative changes compared to control groups with no antioxidant [17].

**Table 3.** TBARS values of sausages (mgMA/kg)

Sample	Month 0	Month 1	Month 2	Month 3
C20A0	0.426±0.08 <sup>a,y</sup>	0.109±0.014 <sup>c,z</sup>	0.541±0.179 <sup>a,xy</sup>	0.747±0.155 <sup>a,x</sup>
C20A1	0.192±0.05 <sup>b,y</sup>	0.206±0.013 <sup>a,y</sup>	0.742±0.191 <sup>a,x</sup>	0.541±0.149 <sup>ab,x</sup>
C10A0	0.251±0.013 <sup>b,yz</sup>	0.144±0.026 <sup>b,z</sup>	0.539±0.089 <sup>a,x</sup>	0.316±0.148 <sup>c,y</sup>
C10A1	0.227±0.021 <sup>b,y</sup>	0.088±0.006 <sup>c,z</sup>	0.440±0.120 <sup>a,x</sup>	0.345±0.039 <sup>c,xy</sup>

Data are presented as the mean values of replications ± standard deviation. abc: Means with the different letter in the same column are significantly different (p<0.05); Data are presented as the mean values of replications ± standard deviation. xyz: Means with the different letter in the same row are significantly different (p<0.05).

Sensory properties of sausages in terms of oiliness, rancid taste and general acceptability are shown in Table 4. Fat reduction significantly affected the oiliness scores of C10 sausages (p<0.05). At the beginning of storage, panelists did not score a rancid taste in any of the sausages and no significant difference was found in general acceptability of sausages (p>0.05).

**Table 4.** Sensory analysis of sausages

Sausage	Oiliness				Rancid Taste				General Acceptability			
	Storage (month)				Storage (month)				Storage (month)			
	0	1	2	3	0	1	2	3	0	1	2	3
C20A0	6.6 ±0.5 <sup>b,y</sup>	7.2 ±0.4 <sup>a,x</sup>	6.8 ±0.6 <sup>a,y</sup>	6.1 ±0.6 <sup>b,z</sup>	1.6 ±0.5 <sup>a,z</sup>	2.2 ±0.6 <sup>a,y</sup>	2.7 ±0.5 <sup>a,y</sup>	3.2 ±0.8 <sup>a,x</sup>	6.5 ±0.5 <sup>a,x</sup>	6.1 ±0.7 <sup>a,xy</sup>	6.0 ±0.7 <sup>ab,xy</sup>	5.6 ±0.5 <sup>a,z</sup>
C20A1	6.7 ±0.8 <sup>b,x</sup>	7.06 ±0.7 <sup>a,x</sup>	6.9 ±0.6 <sup>a,x</sup>	6.4 ±0.5 <sup>ab,x</sup>	1.5 ±0.5 <sup>a,y</sup>	1.7 ±0.7 <sup>ab,y</sup>	1.9 ±0.7 <sup>b,y</sup>	2.5 ±0.5 <sup>b,x</sup>	6.5 ±0.5 <sup>a,x</sup>	5.8 ±0.6 <sup>a,y</sup>	5.7 ±0.5 <sup>b,y</sup>	5.7 ±0.5 <sup>a,y</sup>
C10A0	7.3 ±0.5 <sup>a,x</sup>	7.2 ±0.4 <sup>a,xy</sup>	7.2 ±0.4 <sup>a,xy</sup>	6.8 ±0.6 <sup>a,y</sup>	1.2 ±0.4 <sup>a,z</sup>	1.7 ±0.5 <sup>ab,y</sup>	2.3 ±0.7 <sup>bc,x</sup>	2.7 ±0.5 <sup>ab,x</sup>	6.5 ±0.5 <sup>a,x</sup>	6.2 ±0.8 <sup>a,x</sup>	6.3 ±0.5 <sup>a,x</sup>	6.0 ±0.5 <sup>a,x</sup>
C10A1	7.5 ±0.7 <sup>a,x</sup>	7.4 ±0.5 <sup>a,x</sup>	7.0 ±0.7 <sup>a,x</sup>	6.9 ±0.6 <sup>a,x</sup>	1.3 ±0.5 <sup>a,y</sup>	1.2 ±0.4 <sup>b,y</sup>	1.6 ±0.7 <sup>c,xy</sup>	1.9 ±0.7 <sup>c,x</sup>	6.3 ±0.5 <sup>a,x</sup>	6.4 ±0.9 <sup>a,x</sup>	6.0 ±0.5 <sup>ab,x</sup>	6.0 ±0.7 <sup>a,x</sup>

Data are presented as the mean values of replications ± standard deviation. abc: Means with the different letter in the same column are significantly different ( $p < 0.05$ ); Data are presented as the mean values of replications ± standard deviation. xyz: Means with the different letter in the same row are significantly different ( $p < 0.05$ ).

Sensory properties of sausages decreased during storage and the lowest general acceptability result of C20A0 sausage was found in the 3rd month ( $p < 0.05$ ) due to higher oxidative changes in this formulation. TBARS results were similar; a more rancid taste was found in C20A0 sausage, which had the highest TBARS values at the end of storage. Reduced fat and  $\alpha$ -tocopherol-added sausages (C10A1) had the lowest rancid taste scores at the end of storage ( $p < 0.05$ ). However, all sausages had similar general acceptability scores at this time ( $p > 0.05$ ). It can be stated that the use of antioxidant in reduced fat sausage produced significant effect on delaying rancid taste development ( $p < 0.05$ ).

#### 4. Conclusion

Since high fat content is related to some health problems, formulating meat products with lower fat content could be a good strategy. In this study, it was seen that lowering the fat content by 50% (so they contained just 10% fat) and adding  $\alpha$ -tocopherol to sausages retarded the oxidative changes at the beginning and at the end of the storage. Even though fat has important effects on sensory properties of meat products, fat reduction did not affect the general acceptability of sausages at the end of storage. However, similar to oxidation results, the addition of  $\alpha$ -tocopherol inhibits rancid taste development in sausages containing just 10% beef fat.

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# A comparative study of fat replacers in cooked sausages

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**Abstract.** Reduction of fat in meat products is an important task aimed at solving the problem of excessive fat intake. Different substances are used as fat replacers: plant and animal proteins, and polysaccharides. The aim of the research was a comparative study of the effect of different fat replacers (inulin; a mixture of hydrocolloids; soy protein, and; collagen protein) on the quality of low-fat cooked sausage. In the experimental samples, 50% of backfat was replaced with: inulin gel (exp. 1), a mixture of hydrocolloids (carrageenan, xanthan gum and guar gum) with additional incorporation of water (exp. 2), hydrated soy protein (exp. 3), or hydrated collagen protein (exp. 4). Addition of the fat replacers reduced the fat content by more than 40%. The use of hydrocolloids and soy protein in the hydrated form as fat replacers negatively affected sausage taste and consistency. Addition of the hydrated animal protein had no significant effect on taste, color characteristics, or water activity, but led to a decrease in the sausage shear force. The sausage produced with inulin had the organoleptic, color and strength characteristics closest to the control sausage. Inulin gel, therefore, is recommended as a fat replacer in cooked sausages.

## 1. Introduction

The problem of high fat intake has gained a considerable importance in the world due to the increased incidence of cardiovascular diseases and development of obesity. An important measure aimed at solving this problem is increasing the production of products with reduced fat contents. Substances with a structure different from fat, for example, protein- and carbohydrate-based substances, can be used as fat replacers separately or in different combinations. Moreover, these components should have properties that allow them to imitate several functional characteristics of fat and impart the necessary tenderness consistency and attractive appearance to a product. When choosing fat replacers, the fact that fat determines product consistency and palatability must be taken into consideration.

Various hydrocolloids are used in reduced fat products: carrageenan [1], guar [2], xanthan gum [3] and others [4, 5]. With that, a prerequisite is maintenance of the typical organoleptic properties of a product.

Soy protein is used as a protein component that allows the fat content in meat products to be reduced, while improving consistency of sausages with reduced caloricity [6], increasing stability of meat emulsions [7], making the structure firmer and retarding oxidation in low-fat pork patties [8].

A promising substance for production of meat products with reduced fat content is inulin, which is closest to the fat components by appearance, texture and taste. Inulin is easy to use and improves the structure of the finished product; its application does not require changes in the production process parameters. In certain cases, the use of inulin also leads to an increase in product yield. It is possible to use inulin in different types of meat products such as cooked sausages [9], and minced meat [10].

When choosing an optimal fat replacer, it is necessary to take into consideration the meat product type. In Russia, the prevalent product type is cooked sausages, which account for about 60% of total volume. In this regard, the aim of this research was to study the effect of different fat replacers on the quality of low-fat cooked sausages.



## 2. Materials and methods of investigation

Cooked sausages were made, with the control containing 56 kg beef, 24 kg backfat, 20 kg water, curing ingredients (common salt, sodium nitrite), spices, food-grade phosphates, and sodium ascorbate.

In the experimental sausages, 50% of the backfat was replaced with: inulin gel with an inulin:water ratio of 1:1.5 (exp. 1), 0.5 kg carrageenan:xanthan:guar (2:1:1) and 14.5 l water (exp. 2), hydrated soy protein with a soy protein:water ratio of 1:5 (exp. 3), or hydrated collagen protein with a collagen protein:water ratio of 1:15 (exp. 4). Cooked sausages were produced by traditional technology. Sausages were cooked to an internal temperature of  $72 \pm 2$  °C to obtain the final products.

The mass fraction of protein in the sausages was determined by mineralization of the Kjeldahl sample using a Foss Tecator Kjeltac 2300 (Foss A/S, Denmark), and nitrogen was determined according to the amount of ammonia formed.

Fat content was determined by extracting the total fat with hexane or petroleum ether with a boiling point of 50 to 60 °C in the Soxhlet extraction apparatus (BUCHI Labortechnik AG, Switzerland).

The mass fraction of carbohydrates was determined by subtracting the values of moisture, fat, protein and ash mass fractions from 100 g of the product.

To determine the caloric value, the conversion coefficients for protein and carbohydrates was 4 kcal/g, while for fat it was 9 kcal/g.

Determination of the meat product color characteristics in the CIELab system was carried out using a spectrophotometer (Spectroton, Russia) while simultaneously measuring reflection coefficients of sausage samples at 24 fixed wavelengths in increments of 13 nm in the visible spectral range from 380 to 720 nm, followed by mathematical processing of the measurement results by the microprocessor controller integrated in the measuring unit.

The shear force was determined using an Instron-3342 universal testing machine (Instron, USA), with subsequent recording and export of results to an Excel file.

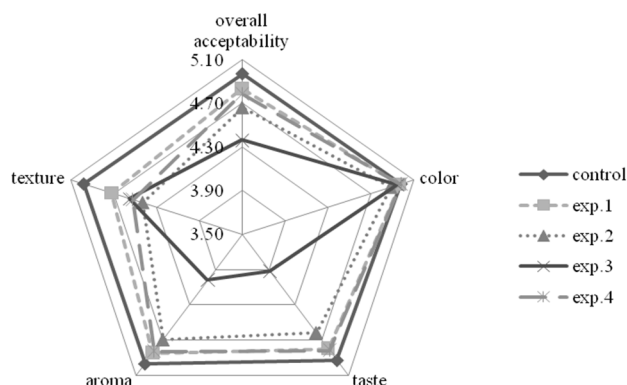
The pH was measured by a potentiometric method using a Zamer-1 portable pH-meter (Zamer, Russia).

The water activity was determined by the cryoscopy method using a Kriometer AWK-20 (Nagy-instruments, Germany).

Each experiment was carried out in three replications. Statistical data processing was performed using Microsoft Excel. The statistical significance of differences between indicators was assessed using the Student's t-test.

## 3. Results of the investigation

The organoleptic assessment of cooked sausages showed that all sausages had good marketable appearance and elastic consistency. The results of the organoleptic assessment are given in Figure 1.



**Figure 1.** Organoleptic assessment of cooked sausages

Addition of hydrated soy protein instead of backfat led to deterioration of cooked sausage taste, which significantly affected the overall score of product acceptability. According Goldman and Brown, the scores for texture and pork flavor of meat patties decreased significantly as soy hulls were added from 0 % to 4 % or 6 %. Off-flavor scores were significantly higher for patties containing 6% soy hulls than for patties with no soy hulls [11]. Yeung and Huang reported soy protein had negative effects on sensory acceptance of emulsified pork meatballs [12]. In our study, the use of hydrocolloids instead of backfat negatively influenced sausage taste and consistency ( $p < 0.05$ ). The cooked sausages in which part of the backfat was replaced with inulin and collagen protein were the closest to the control by color, taste and odor. Some analogous results have been published by Vasilev et al., who stated that up to 8 % inulin gel can be added to sausage without significant effect on sensory properties [13]. Choe et al. indicated no significant differences in color, flavor, tenderness, juiciness, warm-off flavor, and overall acceptability between control sausage and sausage containing 20% pig skin and wheat fiber mixture [14]. However, the sausage with collagen protein in the current study was significantly inferior to the control ( $p < 0.05$ ) in terms of consistency.

Table 1 presents the results of the determination of the cooked sausage chemical composition. The use of fat replacers instead of backfat did not affect the protein content in the cooked sausages ( $p > 0.05$ ).

**Table 1.** Chemical composition of cooked sausages

	Mass fraction in the product, g/100 g sausage			Caloricity, kcal
	Protein	Fat	Carbohydrates	
<b>Control</b>	11.9±0.3	25.1±1.4	-	273.5
<b>Exp. 1</b>	11.3±0.2	14.8±0.3*	4.8±0.2*	197.6
<b>Exp. 2</b>	11.0±0.2	14.1±1.1*	0.4±0.0*	172.5
<b>Exp. 3</b>	12.5±0.3	14.6±0.5*	-	181.4
<b>Exp. 4</b>	11.7±0.3	13.9±0.4*	-	171.9

\* $p < 0.05$  compared to control

The fat mass fraction in the experimental sausages was 41.0–44.6 % lower ( $p < 0.05$ ) compared to the control. Incorporation of the fat replacers resulted in products with the fat content reduced by more than 40 % and caloricity reduced by 27–37 % compared to the control. Vasilev et al. noticed that the addition of inulin reduced the fat content in functional cooked sausages compared to traditional ones [13]. According to Choe et al., fat content decreased by 40 % with addition of 15% pig skin and wheat fiber mixture compared to the control, and the caloric content was 19.0–31.9 % lower in sausage samples containing pig skin and wheat fiber than that of the control [14].

The use of the fat replacers did not significantly affect water activity, pH or color characteristics of cooked sausages ( $p > 0.05$ ) (Table 2). Similar data were obtained by Alvarez and Barbut, who found no differences in color of sausages with up to 6% inulin gel [15]. Modi et al. reported the different levels of carrageenan used in low-fat meat kofta had no effect on  $a^*$  and  $b^*$  values [16]. Silva-Vazquez et al. indicated no differences between  $a^*$  values of traditional sausages and treatment with 30 % inulin [17]. Choe et al. noticed the color values of cooked frankfurter-type sausages containing pig skin and wheat fiber were not affected by the fat content [14]. In contrast, according to Rather et al., high-fat goshtaba had a significantly higher  $L^*$  value, but a lower  $a^*$  value than its low-fat counterparts [18].

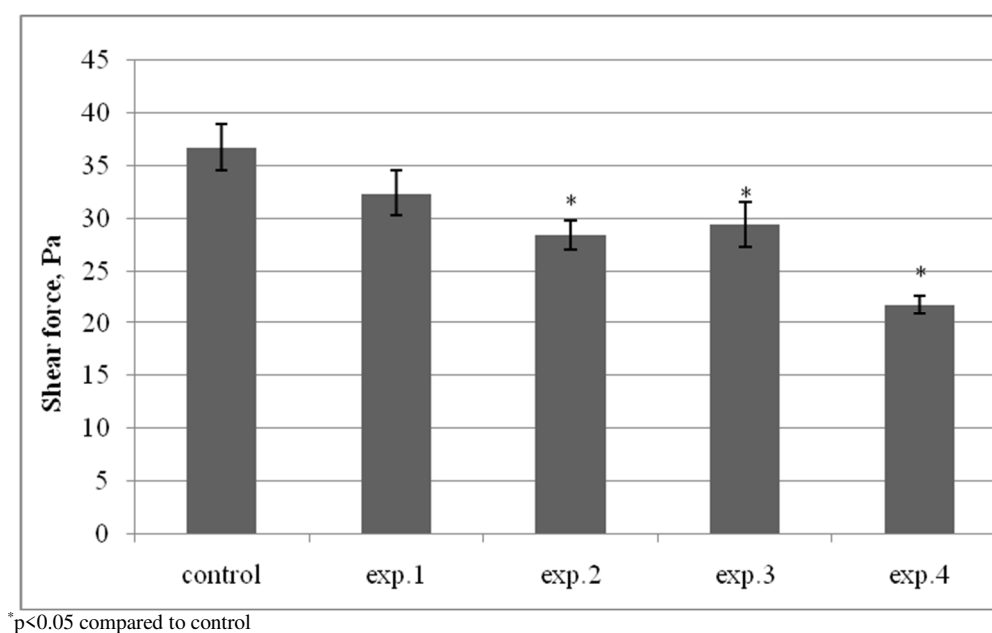
**Table 2.** Physico-chemical properties of cooked sausages

	Indicators				
	Water activity, units	pH	Color characteristics		
			lightness $L^*$	redness $a^*$	yellowness $b^*$
<b>Control</b>	0.9778±0.0009	6.81±0.04	59.3±1.2	15.2±0.6	9.9±0.2
<b>Exp. 1</b>	0.9790±0.0011	6.89±0.01	57.9±0.7	15.6±0.2	10.0±0.1

<b>Exp. 2</b>	0.9810±0.0003	6.92±0.01	59.2±1.5	15.1±0.4	10.2±0.3
<b>Exp. 3</b>	0.9805±0.0013	6.93±0.02	58.2±1.3	15.8±0.9	9.6±0.1
<b>Exp. 4</b>	0.9798±0.0004	6.94±0.02	58.6±1.2	16.1±0.6	9.9±0.4

p>0.05 compared to control

Decreases in the back fat content of the cooked sausages affected product texture. The results of structural-mechanical investigations are presented in Figure 2.



**Figure 2.** Shear force of cooked sausages

The use of the fat replacers facilitated decreases in the shear force compared to the control, which was apparently linked with the additional incorporation of moisture into the product composition. The shear force value closest to the control was in sausage produced with the inulin gel (exp. 1; p>0.05). Some analogous results have been published by Modi et al., who indicated the hardness of cooked and fried low-fat meat kofta decreased with the increase of carrageenan levels [16]. Alvarez and Barbut reported that the addition of inulin resulted in a creamy and softer product [15]. In contrast, according to Keenan et al., the hardness increased with increasing inulin concentration [19]. Goldman and Brown found as soy hulls were added to ground pork, peak force values decreased [11]. Ulu noticed when fat level was decreased, hardness decreased in both raw and cooked meatballs [1]. Similar data were obtained by Rather et al., who indicated the hardness was significantly greater in high fat restructured meat product in comparison with low-fat ones [20].

#### 4. Conclusions

The use of fat substitutes instead of backfat in cooked sausages led to a decrease in fat by more than 40%, without significantly affecting the physico-chemical properties of pH, water activity, and color characteristics. The decrease in fat in cooked sausages was accompanied by a decrease in hardness. Organoleptic indicators and shear stress depended significantly on the type of fat substitute used. Addition of soy protein and hydrocolloids instead of backfat in the cooked sausage resulted in product with reduced fat content and lower caloricity, but it negatively affected the organoleptic properties of product taste and consistency. The use of the inulin gel and collagen protein as fat replacers resulted in production of cooked sausages with low fat content and high overall acceptability. However, sausages with collagen protein were significantly inferior to the control in terms of consistency, while those with inulin had organoleptic and



physico-chemical properties closest to the control. Therefore, this comparative investigation of the stabilizers, polysaccharide and natural protein, shows inulin to be an optimal fat replacer in these cooked sausages.

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# Testing animal feed for the presence of ruminant DNA using the official real-time PCR method

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**Abstract.** Feed has become liable to testing for the presence of ruminant DNA as part of the eradication process of transmissible spongiform encephalopathies. For safety reasons, by the so-called total feed ban, meat and bone meal has been excluded from the diet of food chain animals for years. However, changes in EU regulations that led to relaxation of this ban began from mid-2013 with the introduction of animal proteins derived from poultry and pigs in feed for aquaculture. The EU published an approved PCR method for determining the species of animals from which the ingredients originate. The EU also validated a real-time PCR protocol for detection of prohibited ruminant DNA. As a result of harmonization with the EU legislation, in 2016, amendments to the Serbian TSE regulations were published and an identical method of feed control prescribed. In the same year, this procedure was implemented by the Institute of Veterinary Medicine of Serbia and accredited to the required standard, so adequate feed control started. The aim of this paper is to present the results of the first 50 animal feeds or feed materials tested for the presence of ruminant DNA using the official real-time PCR method. A high percentage (54 %) of the 50 feeds/feed materials studied contained ruminant DNA.

## 1. Introduction

Processed animal proteins (PAP) for use in animal nutrition are largely obtained as by-products of dairies, slaughterhouses and the fish processing industry. These feedingstuffs are characterized by a high percentage of proteins (over 50 %, or some types even close to 80 %). They all possess significant biological value due to their ideal amino acid composition. Consequently, especially in developed countries, PAP was often used in the feeding of highly productive animals. However, after the outbreak of bovine spongiform encephalopathy (BSE), commonly known as mad cow disease and diagnosed in the UK in 1986, it was found that this prion spread *via* feed, if the infectious ruminant proteins were processed into meat and bone meal (MBM). Eradication started immediately and one of the most important measures was to pass regulations to exclude entry of these nutrients into the food chain [1,2].

EU Regulations 999/2001 [3] and 1234/2003 [4] prohibit the application of PAP, which include the various types of MBM, for all farm animals entering the human food chain, except fishmeal to non-ruminants. Also, regulation 1774/2002 [5], repealed by regulations 1069/2009 [6] and 142/2011 [7], prescribed general guidelines for the safe use of by-products of animal origin and prohibited the use of proteins originating from the same species in animal nutrition. Such strict measures were introduced because the incriminated ingredients can potentially cause prion infections not only in animals, but also indirectly in humans, through their consumption of food of animal origin.



On the other hand, a total ban on MBM in the diet of farm animals, although very successful in terms of eradication of transmissible spongiform encephalopathies (TSE), is a waste of highly valued proteins, which has brought great losses in the economic and environmental sense. Annual production of animal by-products from the meat, milk and egg supply in the EU is about 17 million tons. Therefore, not only because of the nutritional importance, but also in terms of sustainability, re-introduction of PAP in the diet of farm animals would have numerous advantages. Also, it is estimated this step would provide an annual profit of about € 350 million [8].

The control system in Serbia, in comparison with the countries of the European Union, was somewhat more complex, with differences in regulations and applied preventive measures. In 2001, amendments to the regulation on quality and other requirements for feed [9] officially banned animal by-products in the diet of ruminants for the first time, while animal by-products were still allowed in diets for monogastric animal species. Identical measures were prescribed by regulation on the quality of feed [10], which came into force on 1<sup>st</sup> of May 2010. However, the regulation on determining, diagnosis and preventing transmissible spongiform encephalopathies [11], from 1<sup>st</sup> of April 2011, introduced a total ban of MBM for all farm animals, equivalent to the ban in Europe. Such a partial feed ban, although preferable for economic and nutritional reasons, enabled cross contamination of feed for ruminants with prohibited ingredients. Therefore, according to article 110 of the Veterinary Law in Serbia [12], feed producers were obligated to separate lines for ruminant feed, or to dispose of MBM and fish meal. Control of feed for the presence of MBM and fishmeal using classical microscopy, as prescribed by EU regulations, started in 2006. From 2011, this monitoring has become a part of the annual state program.

However, changes in EU regulations and relaxation of the ban began from mid-2013 with the introduction of animal proteins derived from poultry and pigs in feed for aquaculture. In addition to classical microscopy, EU Regulation 51/2013 [13] approved a PCR method for detecting the species of animals from which the ingredients originate. The EU Reference Laboratory for Animal Proteins in Feedingstuffs also validated a real-time PCR protocol for detection of prohibited ruminant DNA. As a result of harmonization with the EU legislation, on the 1<sup>st</sup> of April 2016, amendments to the Serbian TSE regulation [14] were published, and an identical method of feed control was prescribed. In the same year, this procedure was implemented by the Institute of Veterinary Medicine of Serbia and accredited to SRPS ISO/IEC 17025 standard, so adequate feed control started. The aim of this paper is to present the results of the first 50 animal feed samples tested for the presence of ruminant DNA using the official real-time PCR method.

## 2. Materials and methods

Altogether, 50 samples of MBM (declared as pure poultry, pig or without the declaration) or fish feed and similar products of animal origin were tested. The applied method was the one prescribed by Serbian TSE regulation [14] and described in detail on the web site of the EU Reference Laboratory for Animal Proteins in Feedingstuffs (EURL-AP). It is presented in the form of an official SOP and protocol [15].

## 3. Results and discussion

Following the European legislation, regular feed analysis started in Serbia soon after implementation of the new real-time PCR protocol. Results of the first 50 tests, grouped according to feed/feed material type, for the presence of ruminant DNA are presented in Table 1.

**Table 1.** Presence of ruminant DNA in 50 animal feeds or feed materials from Serbia

Type of animal feed/material	Number (%) of positive feeds/materials	Number of negative feeds/materials
Poultry MBM	18 (62)	11
Pig MBM	0	1

MBM without declaration	8 (89)	1
Fish feed	1 (11)	8
Fish meal	0	1
Hemoglobin powder	0	1
<i>Total</i>	<i>27 (54)</i>	<i>23</i>

From the data given in Table 1, it is obvious that numbers and percentages of animal feeds/feed materials containing ruminant DNA was high. As we observed with the start of microscopic examination [2], it takes some time for new laws to be fully accepted. Besides that, the PCR method is very sensitive and detects extremely low levels of contamination. It is an efficient way to copy small numbers of DNA segments, and theoretically, one copy can be amplified. Intentional adulteration or attempted fraud is even easier to discover. Therefore, when this is understood and accepted, a downward trend should be expected for the presence of ruminant DNA in animal feeds/feed materials where it is not allowed. Those feeds/feed materials with confirmed positive results showing they contain ruminant DNA can only be used in pet and fur animal feeds.

The introduction of PCR as the official method and the validation of a PCR assay for the detection of ruminant DNA in feed allowed the re-authorization of non-ruminant PAP in feed for aquaculture. It is important to emphasize that according to the present operational scheme, PCR should be used in combination with light microscopy for analysis of feed or feed material for aquaculture. The operational protocol for the analysis of feed or feed material intended for farmed animals other than aquaculture animals and fur animals (e.g. feed for ruminants, pigs, poultry, horses, rabbits,...) includes only the light microscopy method [16]. For this reason, excellent knowledge of the current regulation and SOP is very important for choosing the method to be applied. Otherwise, the wrong choice of method can give a result that is unusable in practical circumstances. Moreover, it can lead to wrong conclusions and can cause problems (RASFF notifications, recall and withdrawal of batches, financial losses and, ultimately, possible health consequences) in the later phases of control or use of feed.

The next steps will be use of poultry PAP for pigs and pig PAP for poultry, as validation of adequate PCR protocols that would allow efficient control are on the horizon. Due to interference of authorized animal ingredients (e.g. fats, blood products, dairy products) with PCR results, additional analytical approaches will probably be needed. In addition to its own research, in 2014, the EURL-AP initiated an international laboratory network to investigate and develop alternative techniques, such as aptamers, mass spectrometry and ELISA. The most promising method is probably mass spectrometry [17]. Recently the identification of proteins and peptide biomarkers allowing the detection of PAPs by mass spectrometry produced very interesting results, but efforts must be continued and the journey to validation and implementation by the control laboratories is long.

Furthermore, the implementation of a third category of animal material in addition to terrestrial, i.e., the terrestrial invertebrates, is proposed [18]. According to novel data, insects are a promising feed and food protein source, but future research needs to provide some solutions before they can be widely utilized in food and/or feed [19]. It is important to fill the existing analytical gaps and to detect the species of interest rapidly. In that regard, the most promising approach today is real-time PCR.

The design of laboratory methods should serve for monitoring authenticity, control of labels/declarations and detection of fraud [20]. Especially when considering the future relaxation of the ban, however, the possibility of cross-contamination remains the biggest threat. Strict avoidance of these undesirable cases (unauthentic, undeclared, unlabeled, fraudulent or cross-contaminated feeds) will remain an obligation for all participants in the food chain.

#### 4. Conclusion

Testing of feed for the presence of ruminant DNA using the official real-time PCR method is no longer new. Over time and by understanding the need for these analyses, participants in the food chain should begin to take advantage of timely detection of unauthorized ingredients. For full effect, laboratories play an extremely important role. Their knowledge of current regulations and SOPs is

crucial for choosing the method to be applied. Otherwise, mistakes at this stage can produce results that are not usable in practical circumstances. Moreover, they can lead to wrong conclusions and cause many problems in later phases of control or use of feed. Therefore, it is essential that feed producers and feed material importers ensure full compliance with legislation and use a functional system of hazard analysis and critical control points. To prevent cross-contamination or intentional adulteration, regular and routine control of raw materials and complete feeds is extremely advisable.

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# Artificial neural network technologies as a tool to histological preparation analysis

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**Abstract.** Food fraud has become one of the great internal and external threats nowadays. For effective and objective detection of adulterated products, real-time control systems are used. Microstructural analysis is one of these methods. It allows rapid quality and composition assessment of food's raw materials and the finished products. Human skills and expertise/involvement in interpreting the results of microstructural analysis is still absolutely necessary, despite the possibility this is subjective. Modern hardware/software systems for histological analysis with neural network technologies will exclude the subjectivity of that result interpretation. For convolutional neural network operating, the "learning with the teacher" model is used. With this training method, a set of data is prepared, which acts as a series of observations, and for which the values of the input and output variables are indicated, such as: histological analysis data > conclusion about the sample composition > adulteration definition. The network learns to establish connections between them. This paper presents the classification parameters for vegetable food component identification as a part of meat raw materials and finished products. A unified database structure has been developed for structuring and summarizing the main microstructural characteristics of histological sections for various types of meat raw materials. The production-rule systems of "If ... then ... else" are given, and vegetable protein components were chosen as an example. Based on these rules, training of convolutional neural network occurs.

## 1. Introduction

Rapid assessment of the raw materials actually used in a meat product is possible based on the structural microscopy of meat components. Histological analysis is still carried out in laboratories by trained histologists utilizing hardware/software systems for histological studies. However, they do not meet all user requirements. Human involvement is absolutely required in interpreting the results. Therefore, the data obtained can be subjective. Moreover, a lack of skilled histologists prevents application of histological assessment in food enterprises. This disadvantage, together with the frequent non-compliance of those laboratories that do meet the requirements, prevents wide application of the microstructural analytical method. As a result, the assessment can often give unreliable and biased data on the raw materials and finished product composition.

To improve the objectivity of food control, implementation of high-performance intelligent technologies is necessary. Automation of microscopy (in the analysis of histological sections) and processing the results obtained will improve the quality and ergonomics of histologist analysis and will allow a database of the accumulated research experience to be compiled.

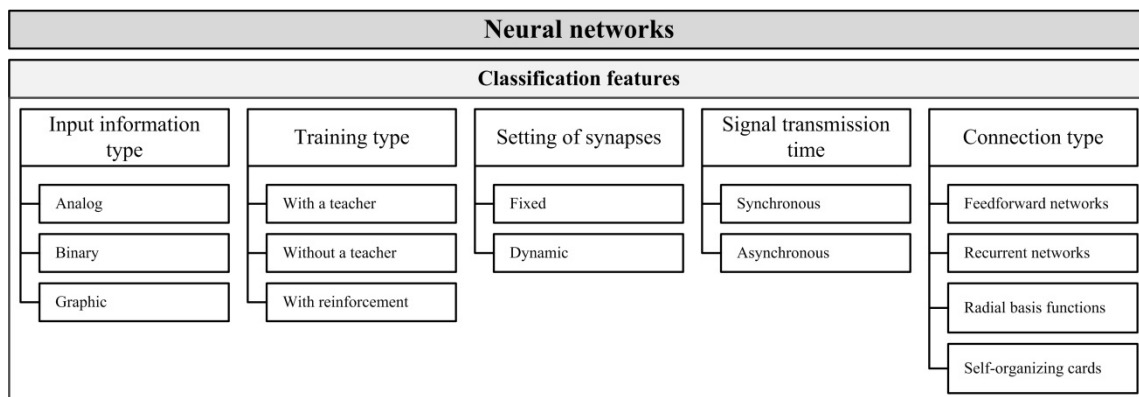




Intelligent systems are technical or software systems capable of solving problems that are traditionally considered to be in a specific knowledge area, data of which are stored in the memory of the system. Intelligent system structure includes three main blocks, i.e. the knowledge base, the decision-making mechanism and the intelligent interface[1]. In decision-making technologies, an intelligent system is an information and computing system with intelligent support that solves problems without involvement of a human decision maker.

Currently, artificial neural networks (ANNs) are the most promising algorithms with respect to the full variety of basic principles on which intelligent systems are built. Solutions based on ANNs show the best results in the field of image recognition and classification compared to other existing algorithms. An ANN analyzes information and assesses whether the available data is consistent with the characteristics that it has learned to recognize. The principal difference of such neural network technologies in information processing is that instead of strictly algorithmic, step-by-step data analysis and calculation of program coding, parallel processing of the whole information array and the drawing up of a training set is used.

ANNs are used to identify and classify information in the case of limited, incomplete and non-linear data sources. When developing ANNs, the main goal is to minimize the direct influence of human researchers on the process of solution finding. Thus, the main advantage of an ANN is the potential ability to develop its own solutions. The use of ANNs makes it possible to eliminate the most difficult part of problem solving, i.e. problem characterization and building the mathematical representation, by training the network on experimental data. Thus, it is possible to avoid idealization of the results. ANNs can be classified as shown (Figure 1) [1].



**Figure 1.** Classification of artificial neural networks

ANNs are classified as follows:

- by the input information type:
  - analog ANNs – use information in the form of real numbers;
  - binary ANNs – operate with information presented in binary form;
  - graphic ANNs – operate with information presented in the form of images: signs, hieroglyphs, symbols.
- by the training type:
  - learning with a teacher, i.e. the output space of neural network solutions is known;
  - learning without a teacher, i.e. the output space of neural network solutions is formed only on the basis of input effects. Such networks are called self-organizing;
  - reinforcement learning, i.e. a system of penalties and rewards from the program environment.
- by the setting of synapses (weights):
  - with fixed connections (weights are selected immediately based on the problem status while  $\frac{dW}{dt} = 0$ , where W are weights of the network);

- with dynamic connections (synapses are adjusted to the process of learning, so  $\frac{dW}{dt} \neq 0$  ).
  - by the signal transmission time:
    - synchronous, i.e. the transmission time of each connection is either zero or a fixed constant;
    - asynchronous, i.e. the transmission time of each connection between elements is different, but constant.
  - by the connection type:
    - Feedforward networks. All connections are directed strictly from the input neurons to the output ones. Examples of such networks are the Rosenblatt's perceptron, the multilayer perceptron, the Ward's networks;
    - Recurrent neural networks. The signal from the output neurons or the neurons of the hidden layer is partially transmitted back to the inputs of the neurons in the input layer (feedback). The Hopfield's recurrent network "filters" the input data, returning to a steady state and, thus, allows problems of data compression and associative memory build-up to be solved.
    - Radial basis functions. Also known as RBF networks. Radial basis network is characterized by three features: single hidden layer; only neurons of the hidden layer have a non-linear activation function; the synaptic weights of the connections in the input and hidden layers are equal to unity.
    - Self-organizing cards. Competitive neural network without a teacher that solves the problems of visualization and clustering.

The choice of the network type (Rosenblatt's perceptron, Hamming's network, Kohonen's network, convolutional neural network, fuzzy multilayer perceptron, etc.) depends on the task. When solving problems of image recognition and classification, a special case of which is a histological section (to determine the microstructure of the sample), convolutional neural networks and multilayer perceptrons are most often used, while Kohonen's network is more often used to do data categorization [2, 3]. Perceptron (lat. perceptio - perception) is a mathematical or computer model of information perception by the brain (cybernetic brain model) proposed by Frank Rosenblatt in 1957 and first implemented as Mark-1 electronic machine in 1960. Perceptron was one of the first neural network models, and Mark-1 was the first neurocomputer in the world.

The nature of network learning depends on the network type chosen. The "learning with the teacher" model is used to work with the convolutional neural network. Using this training method, a set of data is prepared as a series of observations, with values of the input and output variables indicated (histological analysis data > conclusion on the sample composition > adulteration definition). The network learns to establish connections between them.

The purpose of this work was to determine the main microstructural characteristics (classification parameters) for the identification of vegetable components in meat raw materials and finished products. An information base of the numerical classification parameters was formed, which is necessary for the ANN in automatic mode to learn how to recognize the study materials.

## 2. Materials and methods

The materials studied were vegetable components of protein and carbohydrate nature, and samples of meat raw materials and finished products (boiled and smoked sausages, delicatessen products). Histological preparations of these studied components were 14  $\mu\text{m}$  thick sections, prepared with a MIKROM-HM525 cryostat (Thermo Scientific), then placed on Menzel-Glaser slides (Thermo Scientific), stained with Ehrlich hematoxylin and 1% aqueous-alcohol eosin solution (BioVitrum), and transferred into glycerin-gelatin according to the standard technique. Histological preparations were studied using an AxioImager A1 light microscope (Carl Zeiss, Germany). Images were obtained using AxioVision 4.7.1.0 image analysis system (Carl Zeiss, Germany).

## 3. Discussion

Automation of visualization processes is convenient and useful. However, the question remaining is how to implement ANN in histologists' workflows with the greatest efficiency. We do not want to

repeat the mistakes of the Digital Pathology Association, which were made several years ago when using automatic diagnosis [4]. Statistical analysis of this method implementation in mammography revealed many errors of the first kind (erroneous deviation from the null hypothesis). For example, according to a study by Korean scientists [5], in medical examinations, the number of first-kind errors (i.e. erroneous detection) in computer diagnostics of mammography data was about 70%. This meant that in the diagnosis of healthy mammary glands, non-existent tumors were detected in more than half of the cases. Palazzetti et al.[6] showed that in the diagnosis of breast malignant tumors, in more than half of the cases (250 patients with cancer and 250 healthy women took part in the study), symptoms of the disease were not noticed by specialists. Therefore, we need to be very careful in choosing the ANN architecture and its training method. The main task is to facilitate the work of histological specialists and help them to draw conclusions (on adulteration/not adulteration), and not to hinder their work.

Thus, in the analysis and classification of images, neural networks showed only moderate results, as noted above, until a new ANN architecture, i.e. convolutional neural network (CNN) was developed. Many people associate CNN with computer vision. CNNs achieved great success in image recognition due to the fact that they are arranged like the visual cortex of the brain, i.e. they can focus on a small area and highlight important features in it. CNN operation is usually interpreted as a transition from specific features of the image to more abstract details, and then to even more abstract details, up to highlighting high-level concepts. At the same time, the network is self-adjusting and produces the necessary hierarchy of abstract features (a sequence of feature maps) by filtering out unimportant details and highlighting important ones.

An algorithm for effective breast cancer CNN-based diagnosis was recently developed at the University of Nijmegen (Netherlands)[7]. Stanford University collected a database of more than 40,000 x-ray images of injured limbs in January 2018. With this database, a neural network was trained and subsequently proved its effectiveness in determining limb injuries comparing to a professional radiologist [8]. Verily Life Sciences (Alphabet Holding) has developed an algorithm that determines the age, gender, and various medical indicators (for example, blood pressure or body mass index) of patients by analyzing their retina [9]. Such a diagnostic method could help in cardiovascular disease prediction. Large databases have contributed to the development of deep learning algorithms, which in such tasks as detection of diabetic retinopathy [10], skin cancer [11], cardiac arrhythmias [12], cerebral hemorrhage [13], pneumonia [14], and hip fractures [15] ensure efficiency at the expert level.

Data on the basis of which ANN builds answers can be of various types and formats: terms describing any situations, numbers or values, graphs, two- or three-dimensional images, etc. Neural network training consists of several stages. Data selection for the network training and their processing is the most difficult step in solving the task. The training data set must meet the criteria of representativeness and consistency.

For detection of the main microstructural characteristics (classification parameters) of identification, we developed a unified database (DB) structure for our histological sections of various meat raw materials and vegetable components present in the composition of meat raw materials and finished products. The identification indicators used were the following [16]: particle shape, size, particle tinctorial characteristics (ability to be stained with histological dyes), soybean shell fragments (for protein components). For example, soybean protein isolate particles (10 to 110  $\mu\text{m}$  size) are stained in pink, soybean protein concentrate particles (30 to 105  $\mu\text{m}$  size) are stained in dark pink to bright red. Starch particles are described as folded strap, or bean, or rounded particle with a dark point in the center. On the other hand, flour particles are orbed and grouped into large aggregates.

The production rules “*If ..., then...*” for identification of vegetable protein components are presented as examples:

*If there are eosinophilic structures, and stained substance predominates in the cell complexes, then soybean protein products are present.*

*If the particles are round, with holes inside, have a homogeneous wall, the shape of donut, dumbbell or flower, then soybean protein isolate is present.*

If the particles are cylindrical (longitudinal section) or rounded (transversal section) cells surrounded by a narrow, non-stained area, i.e. cellulose membrane, *then* soybean protein concentrate is present.

If there is fibrous component, i.e. thin loose fiber bundles and narrow cylindrical cells not stained with common histological dyes, *then* textured soybean protein product is present.

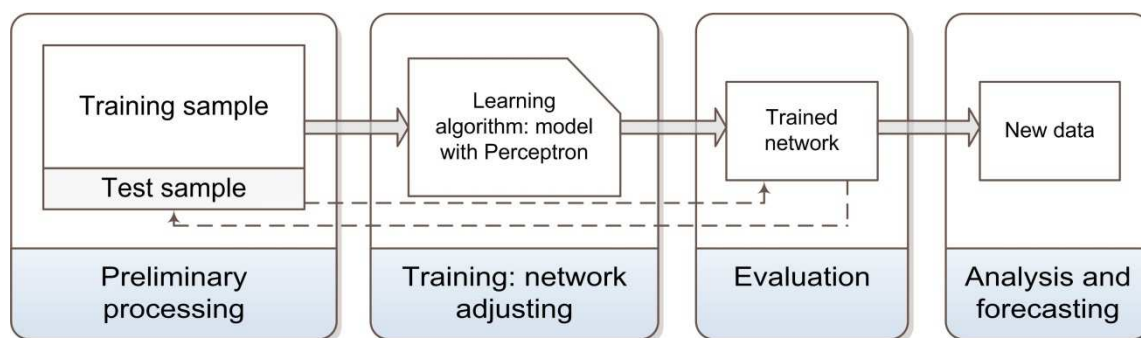
If there are eosinophilic structures of round shape, in which non-stained starch granules are present, *then* pea flour is present.

If the particles are round, with holes inside, have a porous wall, the shape of donut, *then* pea isolate is present.

If there is fibrous component, i.e. thin dense fiber bundles, while there are no non-stained cylindrical cells, *then* wheat textured product is present.

If the structures are not stained, and they look like narrow cylindrical cells, in which only transparent stacked cell membranes are detected, *then* soybean shell cells are present.

The database (set of training pairs) will be divided into two unequal parts. Most of the data will be used for training (training samples), the other part will be used for testing the ANN (test samples). Figure 2 shows our ANN training module.



**Figure 2.** Artificial neural network training diagram

Training data are used to train the network. Test data are used to calculate the errors of the network. However, test data are never used for network training. A test data error decrease confirms the network generalization process. If the error continues to decrease with the training data and increases with the test data, it means the network has stopped performing a generalization and just “remembers” the training data. This phenomenon is called network retraining or overfitting (overtraining (overfitting) in machine learning and statistics is a phenomenon when the constructed model well explains the examples from the training sample, but does not work well with examples that were not used in training (test sample). This is due to the fact that when developing a model (“in the process of training”), some random patterns are found in the training sample, which are absent in the general data array. In such cases, training is usually stopped. In the process of training, other problems can appear, such as “paralysis” or local minimum of the error surface. It is impossible to predict a particular problem, or to give unambiguous recommendations for their resolution.

#### 4. Conclusion

Objective histological results related to food adulteration are possible only when a skilled histologist with appropriate work experience conducts the assessment. The trained specialist does not just analyze the indicators in accordance with generally accepted criteria, but compares them with those many samples they have already seen and analyzed during their work. The higher the qualifications and the histologist’s experience is, the larger is their “photo library” and the greater is the likelihood that the histologist will correctly evaluate the microstructural characteristics of any sample they examine. Thus, the proper result depends on the knowledge, skills, experience and professional competencies of the histologist. However, the use of modern informational approaches to processing and interpreting information facilitates this decision-making process. The use of neural network

technologies for food composition control is a socially significant procedure. It can significantly speed up the process of adulteration detection in laboratories of both governmental control organizations and food processors. It will definitely increase the objectivity of the results obtained, help to eliminate unfair competition in the food market, ensure product meets consumer expectations as regards food content, and ensure consumer protection.

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## Cadmium level trend in liver and kidney of pigs from Serbia during 2014-2018

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**Abstract.** Concentrations of cadmium (Cd) were measured in samples of liver (n=88) and kidney (n=226) of pigs within the Serbian National Residue Monitoring Program during 2014-2018. Levels of Cd were determined by inductively-coupled plasma mass spectrometry. All of the pigs had measurable amounts of Cd in liver and kidney, but none of the analyzed samples exceeded MRL for Cd – 0.5 and 1.0 mg/kg, respectively. The maximum Cd concentration in kidney amounting to 0.864 mg/kg was established in the first year of the residue monitoring program (2014). No statistically significant differences in Cd levels in liver samples during the examined period were observed, while statistical differences were established only between Cd levels in kidney collected in 2014 and 2015. On the basis of statistical analysis, it can be concluded that Cd levels have not increased in the analyzed organs during the investigated period.

### 1. Introduction

Cadmium (Cd) is a toxic heavy metal well known as an environmental and food chain contaminant [1,2]. Water and air currents facilitate its transfer over long distances from sources of pollution, until it enters plants through soil deposition, and consequently animals and humans through nutrition. Industrial development and human activities increase the levels of this naturally occurring element in the environment [3,4]. Diet is the primary source of Cd for the nonsmoker population, while tobacco smoke is the major source for smokers [5]. Cd is not degradable and accumulates in various tissue types, specifically in kidney [6]. After exposure, Cd can damage kidney, liver, and nervous system, can cause diabetes, lead to bone disorder, problems with vitamin D metabolism, and cardiovascular disease, etc. [7-10]. Considering the chronic Cd exposure of humans through diet, along with its ability to accumulate and long half-life in the body [11], Cd poses a significant health risk to humans [12]. According to the International Agency for Research on Cancer (IARC) Cd and Cd compounds are carcinogenic to humans [13], as Cd is pronounced as human lung carcinogen [14]. Given that food is one of the most significant sources of Cd exposure, the European Commission [15] and Serbian national regulation [16] has established maximum residue levels for Cd in muscle, liver and kidney intended for human consumption (0.050, 0.5 and 1 mg/kg, respectively). In order to secure the hygiene of food of animal origin and to protect public health, it is necessary to establish control and monitoring programs encompassing adequate numbers of samples, as well as the efficient monitoring of the residue level in tissues and organs of animals [17].



The objective of this study was to review the Cd levels in liver and kidney of pigs collected during the Serbian monitoring program in the period 2014-2018, and to establish whether levels of Cd in pig tissues are increasing.

## 2. Materials and Methods

The levels of Cd were measured in samples of liver (n=88) and kidney (n=226) of pigs. Analyzed tissues were collected as a part of Serbian monitoring program during 2014-2018. Samples were individually stored in plastic bags at -18 °C prior to analysis.

Amounts of 0.3 g measured with precision of  $\pm 0.001$  g were transferred into Teflon vessels and treated with 5 mL of nitric acid (67% Trace Metal Grade, Fisher Scientific, Bishop, UK) and 1.5 mL of hydrogen peroxide (30% analytical grade, Sigma-Aldrich, St. Louis, MA, USA). Sample homogenates were further treated in the microwave (Start D, Milestone, Sorisole, Italy) according to the following temperature program: 5 min from room temperature to 180°C, 10 min hold 180°C, and 20 min vent. Digested homogenates were quantitatively transferred into volumetric flasks and diluted to 100 mL with deionized water (ELGA, Buckinghamshire, UK).

The analysis of  $^{111}\text{Cd}$  isotope was performed by an inductively coupled plasma-mass spectrometry (ICP-MS) instrument iCap Q (Thermo Scientific, Bremen, Germany), equipped with a collision cell, and operating in the kinetic energy discrimination (KED) mode. A five-point calibration curve (including zero) was constructed for quantification. Multielemental internal standard ( $^6\text{Li}$ ,  $^{45}\text{Sc}$  – 10 ng/mL;  $^{71}\text{Ga}$ ,  $^{89}\text{Y}$ ,  $^{209}\text{Bi}$  – 2 ng/mL) was introduced online via another line through the peristaltic pump. Measured concentrations were corrected for the response factors of internal standards. The quality of the analytical process was confirmed by the analysis of the standard reference material SRM 1577c (Gaithersburg, MD, USA), and were within the range of the certified values.

Statistical analysis was performed using the Minitab 16.0 software. One-way (unstacked) ANOVA analysis of variance and Tukey's test were used in order to compare the differences in Cd concentrations between liver and in kidney from different years.

## 3. Results and Discussion

The Cd levels measured in liver and kidney samples are presented in Tables 1 and 2, respectively. As expected, all analyzed samples were above the limit of detection (LOD = 0.001 mg/kg) since Cd primarily accumulates in liver and kidney, bonding to metallothionein in these tissues [18,19]. Liver had lower mean Cd concentrations than kidney. Also, none of the analyzed samples exceeded the MRLs for Cd in pig liver and kidney (0.5 and 1.0 mg/kg, respectively).

**Table 1.** Levels of Cd in pig liver by year

Year	Mean Cd, mg/kg	min-max
2014	0.022	0.007-0.085
2015	0.025	0.013-0.046
2016	0.024	0.008-0.042
2017	0.025	0.005-0.049
2018	0.030	0.009-0.080

**Table 2.** Levels of Cd in pig kidney by year

Year	Mean Cd, mg/kg	min-max
2014	0.182 <sup>a</sup>	0.014-0.864
2015	0.126 <sup>b</sup>	0.007-0.261
2016	0.135 <sup>ab</sup>	0.038-0.306
2017	0.131 <sup>ab</sup>	0.024-0.351
2018	0.161 <sup>ab</sup>	0.046-0.469

<sup>a-b</sup> Different superscripts indicate significant differences of means according to Tukey's HSD test ( $p < 0.05$ )

Measured Cd levels of all analyzed liver samples were similar to data reported by Jankovic *et al.* (2012) for pig liver (0.033 mg/kg) [1]. They [1] examined Cd levels in offal of cattle, pigs, lambs and horses collected during the Serbian monitoring program from 2011 to 2012. Cd levels determined in kidney samples during 2014 were higher while data obtained in other years (2015, 2016, 2017 and 2018) were similar to Cd levels reported in the previously mentioned study [1]. The mean Cd levels in pig liver of this study were in line with levels reported by Nikolic *et al.* (2013) [20], who analyzed samples of liver and kidney in swine from the Serbian market. Comparing mean Cd levels of kidney from the current study with concentrations determined by [20], the same situation was observed as in data sets established by Jankovic *et al.* (2012) [1], except for Cd levels from 2014. Another study of Nikolic *et al.* (2017) [21] analyzed mineral composition of tissues (muscle, liver and kidney) of intensively and extensively reared pigs. Cd levels from our current study can be compared with data for Cd levels in liver and kidney of swine reared in intensive systems if it is assumed that pigs were kept in comparable conditions and were fed with similar feed. Our results for Cd in liver were lower than those reported by Tomović *et al.* (2011) (0.412 mg/kg) [22] and López-Alonso *et al.* (2007) (0.073 mg/kg) [23]. Also, the mean Cd levels in kidney analyzed during the period 2014-2018 were lower compared to some other previously reported studies [21,23-26].

According to some studies [27-29], Cd levels in feedstuffs strongly influence the Cd levels in liver and kidney of animals, and this could be one of the reasons why the Cd levels measured in the current study were different to data reported in these previously mentioned studies.

#### 4. Conclusion

In summary, all samples of liver and kidney taken from the pigs tested within the Serbian National Residue Monitoring Program during 2014-2018 contained Cd. Liver had lower mean Cd concentrations than kidney. The highest Cd level was measured in kidney (0.864 mg/kg) collected during 2014. No increasing trend of Cd concentrations in the analyzed organs during five years (2014–2018) was observed.

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## An insight into *in vitro* antioxidant activity of *Cantharellus cibarius* hot water extract for the potential application in meat products

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**Abstract.** The current research was undertaken to estimate the *in vitro* antioxidant activity of *Cantharellus cibarius* mushroom extracted by boiling in water for 30 minutes. Several previous studies have shown that the addition of edible mushrooms in meat products affects the reduction of lipid oxidation and prolongs the shelf-life of the final products. Antioxidant capacity of *C. cibarius* was measured using the following methods: reducing power ability, lipid peroxidation assay, cupric ion reducing antioxidant capacity (CUPRAC) and DPPH free radical scavenging activity. Reducing power and antioxidant activity assays of *C. cibarius* hot water extract showed low antioxidant activity. CUPRAC assay demonstrated positive effect only at the concentration of 10 mg/mL, whereas DPPH radical scavenging activity showed moderate antioxidant activity in comparison with culinary-medicinal mushrooms, with the effective concentration (EC<sub>50</sub>) from 7.41 mg/mL.

### 1. Introduction

Mushrooms have various quality characteristics that have been defined by Djekic *et al.* [1] and they are particularly respected for their taste and texture [2]. A plenty of protein, fiber, vitamins and minerals are contained in mushrooms. Typically, dried mushrooms comprise of 22% protein, which contains most of the essential amino acids, 5% fat, mostly in the form of linoleic acid (the essential fatty acid not



synthesized in the human organism), 63% carbohydrates including fiber and 10% good source of minerals counting thiamin, riboflavin, niacin and biotin [3].

Mushrooms have a tendency to gather a variety of secondary metabolites including phenolic compounds, polypeptides, terpenes, steroids, etc. Their phenolic compounds have been found to be an excellent antioxidants [4]. It is very important due to the fact that oxidation is one of the most important processes of food deterioration since it may affect food safety, color, flavor and texture [5].

Antioxidants or molecules with radical scavenging capacity are believed to exert a potential defending effect against free radical destruction. Methanol and/or water extracts from common button (*Agaricus bisporus*), shiitake (*Lentinus edodes*), straw (*Volvariella volvacea*), oyster (*Pleurotus* sp.), winter (*Flammulina velutipes*), ear (*Auricularia* sp. and *Tremella* sp.) mushrooms [6,7] have displayed important antioxidant activities [8]. Presently, not many reports can be found depicting the bioactive effects of wild edible mushrooms usually found in European woods [9].

The chanterelle *Cantharellus cibarius* is broadly viewed as among the most desired of wild edible mushrooms [10]. It is presumably the best known species of the genus *Cantharellus*, if not of the complete family of *Cantharellaceae*. *C. cibarius* is world famous not only as palatable food, but also because of its spreading from Scandinavia to the Mediterranean in Europe [10].

Mushroom decoctions contain the hot water dissolvable components from the fruiting body. Thus, squash or small pieces of the fruiting body are boiled and the descend decoction is consumed. Besides that, mushrooms are generally not eaten raw, but subjected to various food processing procedures in order to be more readily assimilated by digestion [11]. Hence, it can be thought that preparation of hot water extracts simulate cooking conditions - the characteristic manner of how edible mushrooms are consumed or how the product of interest (cooked sausages) are processed. Therefore, the objective of our investigation is to examine whether *Cantharellus cibarius* water decoction exerts antioxidant activity *in vitro*, through the following methods: the reducing power, inhibition of lipid peroxidation, CUPRAC (cupric reducing antioxidant capacity) and the scavenging capacity of the radical DPPH. In relation to this, it will be decided whether this mushroom could be used for the production of cooked sausages in order to potentially extend the shelf-life of the final product.

## 2. Materials and methods

### 2.1. Preparation of mushroom decoction

Milli-Q water, obtained from a Milli-Q water purification system (Merck, Darmstadt) was used. In order to obtain a decoction (hot aqueous mushroom extract), a mixture of dry powdered mushroom and MQ water (1:10) was heated at 100°C, 30 min. The resulting decoction was subjected to the assays determining the reducing power, inhibition of lipid peroxidation, CUPRAC (cupric reducing antioxidant capacity) and the scavenging capacity of the radical DPPH *in vitro*.

### 2.2. Reducing power (FRAP)

The reducing power was determined according to Petrović *et al.* [12].

### 2.3. Lipid peroxidation

Conjugated diene method according to Lingnert *et al.* was used [13].

### 2.4. Cupric reducing antioxidant capacity (CUPRAC)

The ability of samples to reduce cupric ion was determined according to method described by Öztürk *et al.* [14]. Solutions of Cu(II) (10 mM, 0.05 mL), neocuproine (7.5 mM, 0.05 mL), NH<sub>4</sub>Ac buffer (1 M, pH 7.0, 0.06 mL) and serial dilutions of mushroom decoction prepared in MQ water (0.04 mL), were added to

a 96-well microplate and incubated for 1h, at 30 °C. The absorbance was measured at microplate reader (Lab Companion, VM-96, Korea) at 450 nm, against blank solution (water was added instead of sample solution).

### 2.5. DPPH free radical scavenging activity

The method was performed according to Vunduk *et al.* [15]. Extract solutions were prepared in MQ water (Merck, Darmstadt).

### 2.6. Statistical analysis

The data were analysed by one-way ANOVA using the SPSS software version 23 (Chicago, Illionis USA). Differences between the means were compared by Tukey's comparative test. A significance level of  $P < 0.05$  was used for evaluations.

## 2. Results and discussion

### 3.1. Reducing power ability

The capability of mushroom extracts to donate electrons could be assessed using the reducing power assay. In the presence of antioxidants, the  $\text{Fe}^{3+}$ -ferricyanide complex is reduced to the ferrous form,  $\text{Fe}^{2+}$  and the latter can be monitored by measuring the formation of Perl's Prussian blue at 700 nm; higher absorbance indicates better reducing power [16]. The ability of mushroom extract and control (ascorbic acid and butylated hydroxyanisole) to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  at different concentrations are shown in Table 1.

**Table 1.** Reducing power of *C. cibarius* hot water extract

Concentration (mg/mL)	<i>C. cibarius</i>
0.625	$0.031 \pm 0.010^A$
1.25	$0.040 \pm 0.000^A$
2.5	$0.102 \pm 0.021^A$
5	$0.280 \pm 0.000^A$
10	$0.534 \pm 0.022^B$
Positive controls*	
Butylated hydroxyanisole (BHA)	$2.506 \pm 0.054^C$
Ascorbic acid	$2.380 \pm 0.197^C$

Notes: Values are mean  $\pm$  standard deviation. Means in the same column with different capital letters are significantly different ( $P < 0.05$ )

\*Absorbance values for positive controls were measured at the concentration of 0.50 mg/mL.

At the concentration of 0.50 mg/mL, the positive controls that is BHA and ascorbic acid displayed high-reducing ability of 2.506 and 2.380, respectively, which are distinctively higher than that obtained from any *C. cibarius* extracts. Mushroom extracts exhibited a variable reducing capacity and general, the reducing capacity increased with increasing concentration, but only at the concentration of 10 mg/mL, it was significantly higher. In comparison to the other investigations of hot water extracts of mushrooms, Tsai *et al.* [17] claimed that reducing powers of *Agaricus blazei*, *Agrocybe cylindracea* and *Boletus edulis* were determined at 0.83, 0.86 and 1.15 at 5 mg/mL, respectively. It is significantly higher than the absorbance obtained from *C. cibarius* even at the concentration which is twice lower. Other authors reported that hot water extracts from Ling chie exhibited reducing powers of 0.48 and 0.44 at 1 mg/mL and 1.08 and 1.04 at 5 mg/mL, respectively. Also, at 5 mg/mL, *P. citrinopileatus* displayed a high reducing power of 1.10 [18] in comparison to our mushroom which absorbance is about twice lower in

twice higher concentration. Apparently, comparing to the other commercial and medicinal mushrooms, *C. cibarius* showed lower reducing power ability for hot water extracts.

### 3.2. Lipid peroxidation

The results obtained using the conjugated diene method for antioxidant activity are shown in Table 2.

**Table 2.** The ability of *C. cibarius* extract and commercial antioxidants to prevent the peroxidation of linoleic acid

Concentration (mg/mL)	<i>C. cibarius</i>	Ascorbic acid	$\alpha$ -tocopherol
0.1	$0 \pm 0^{a,A}$	$78.33 \pm 0.91^{b,A}$	$82.77 \pm 0.75^{c,A}$
1	$0 \pm 0^{a,A}$	$79.50 \pm 0.75^{b,A}$	$82.88 \pm 0.06^{c,A}$
2.5	$0 \pm 0^{a,A}$	$79.60 \pm 0.9^{b,A}$	$81.53 \pm 0.11^{c,A,B}$
5	$11.75 \pm 1.98^{a,B}$	$80.84 \pm 0.72^{b,A,B}$	$80.94 \pm 0.06^{b,B}$
10	$22.36 \pm 1.34^{a,C}$	$82.73 \pm 0.8^{b,B}$	$82.36 \pm 1.11^{b,A}$

Notes: Values are mean  $\pm$  standard deviation. Means in the same column with different capital letters and means in the same row with different lowercase letters are significantly different ( $P < 0.05$ ).

The conjugated diene method is based on the ability of the substance to slow the oxidation of conjugated dienes, which can be formed only by polyunsaturated fatty acids. At the concentration of 5 and 10 mg/mL, mushroom extract showed low antioxidant activity, while in lower concentration did not show any effect at all. Widely used commercial antioxidants, ascorbic acid and  $\alpha$ -tocopherol expressed high antioxidant activity at the investigated concentrations. Hot water extracts from wild edible mushrooms, such as *Boletus edulis* showed high antioxidant activity (85.7%) at 5 mg/mL [17]. Similarly, at 5 mg/mL, *H. marmoreus* showed moderated antioxidant activities (38.6%) [19]. Regarding the method of slowing the oxidation of conjugated dienes, our mushroom has not achieved the expected effect.

### 3.3. CUPRAC assay

The CUPRAC assay utilized copper (II)–neocuproine (Cu(II)-Nc) reagent as the chromogenic oxidizing agent. It is based on the monitoring of the formation of stable complex between neocuproine and copper (I) by measurement of absorbance at 450 nm [16]. CUPRAC of the mushroom extracts was assessed and compared to that of the positive controls and shown in Table 3.

**Table 3.** CUPRAC of *C. cibarius* and commercial antioxidants

Concentration (mg/mL)	<i>C. cibarius</i>	Butylated hydroxytoluene (BHT)	$\alpha$ -tocopherol
0.1	$0 \pm 0^{a,A}$	$0.90 \pm 0.1^{b,A}$	$0.44 \pm 0.04^{c,A}$
1	$0 \pm 0^{a,A}$	$1.54 \pm 0.13^{b,B}$	$0.69 \pm 0.01^{c,B}$
2.5	$0 \pm 0^{a,A}$	$2.52 \pm 0.02^{b,C}$	$1.11 \pm 0.01^{c,C}$
5	$0 \pm 0^{a,A}$	$3.33 \pm 0.1^{b,D}$	$1.48 \pm 0.08^{c,D}$

10	$1.545 \pm 0.2^{a,B}$	$4.08 \pm 0.04^{b,E}$	$2.06 \pm 0.04^{c,E}$
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Notes: Values are mean  $\pm$  standard deviation. Means in the same column with different capital letters and means in the same row with different lowercase letters are significantly different ( $P < 0.05$ )

Testing the antioxidative ability of mushroom extract using the CUPRAC method, it was observed that only at the concentration of 10 mg/mL *C. cibarius* demonstrated the antioxidative activity. However, it is worth mentioning that antioxidative potential of *C. cibarius* determined in CUPRAC assay for the highest tested concentration, was only 25% lower than the same determined for the model antioxidant  $\alpha$ -tocopherol. Presently, CUPRAC assay is not frequently used among researchers working on antioxidant studies and little has been published on the assessment of cupric ion-reducing ability of mushroom, especially for the hot aqueous extracts [20, 21]. In the study of Abdullah *et al.* [20], the minimum and maximum absorbance from 14 culinary-medicinal mushrooms, at the concentration of 10 mg/mL were in range from  $1.739 \pm 0.222$  to  $2.778 \pm 0.015$ , which is higher in comparison to our result.

### 3.4. Scavenging activity of DPPH radical

One of the most common procedures for determination of antioxidant capacity is the DPPH free radical scavenging activity assay [20]. DPPH assay is based on the measurement of the scavenging capability of antioxidants toward the stable radical DPPH. The DPPH radical is reduced to the matching hydrazine when it reacts with hydrogen donors [22].

The results of *C. cibarius* hot water extract and commercial antioxidants scavenging ability are shown in Table 4.

**Table 4.** Scavenging ability of hot water extract from *C. cibarius* and commercial antioxidants

Concentration (mg/mL)	<i>C. cibarius</i>	Ascorbic acid	Butylated hydroxytoluene (BHT)
0.1	$12.72 \pm 10.02^{a,A}$	$81.33 \pm 1.11^{b,A}$	$5.56 \pm 1.17^{c,A}$
1	$13.43 \pm 2.95^{a,A}$	$83.65 \pm 0.09^{b,B}$	$10.51 \pm 0.70^{a,B}$
2.5	$20.57 \pm 1.85^{a,A,B}$	$83.38 \pm 0.15^{b,B}$	$21.10 \pm 1.00^{a,C}$
5	$26.08 \pm 9.15^{a,B}$	$84.38 \pm 0.26^{b,B}$	$32.93 \pm 1.81^{a,D}$
10	$31.20 \pm 1.10^{a,B}$	$81.07 \pm 0.49^{b,A}$	$54.12 \pm 1.00^{c,E}$

Notes: Values are mean  $\pm$  standard deviation. Means in the same column with different capital letters and means in the same row with different lowercase letters are significantly different ( $P < 0.05$ )

As shown in Table 4, scavenging ability of mushroom extracts was dependent on the concentration of the extract. In comparison to ascorbic acid, *C. cibarius* had significantly lower values for each concentration, whereas with BHT, mushroom exhibited almost identical ability except for the concentration 10 mg/mL, when BHT expressed significantly higher value.

The scavenging activity of mushroom extracts towards DPPH free radicals can also be expressed in term of  $EC_{50}$ .  $EC_{50}$  (mg/mL) is the effective concentration of the mushroom extract that are required to show 50% antioxidant properties. A lower  $EC_{50}$  value corresponds to higher antioxidant activity of the mushroom extract [10]. Puttaraju *et al.* [23] showed that a hot water extract from *C. cibarius* among 23 mushrooms naturally grown in India took 21<sup>st</sup> place with  $EC_{50}$  value 6.40 (mg/mL). Our result (7.41 mg/mL) for  $EC_{50}$  corresponds to the investigation from Puttaraju *et al.* [23] and also it would take the same place among investigated mushrooms. On the other hand, Abdullah *et al.* [20] investigated the

scavenging activity from 14 culinary-medicinal mushrooms and showed the EC<sub>50</sub> effect in the range 5.28-39.05 (mg/mL). Only *G. lucidum* showed better scavenging activity than our mushroom with the value for EC<sub>50</sub> 5.28 mg/mL.

The capability of hot water extract of other mushrooms to quench free radicals has been described earlier. Hot water extracts of mature and baby Ling chih (*Ganoderma tsugae* Murrill) exhibited excellent antioxidant activities with low EC<sub>50</sub> of 0.30 and 0.40 mg/mL, respectively [24]. In their study, Chirinang *et al.* [25] noticed that the radical scavenging activity of water extract of *Pleurotus ostreatus* (EC<sub>50</sub> = 11.56 mg/mL) was better than that of *P. sajorcaju* (EC<sub>50</sub> = 13.38 mg/mL) presumably due to higher content of phenolic compounds and dietary fibres. *Agaricus blazei*, *Agrocybe cylindracea* and *Boletus edulis* displayed moderate DPPH scavenging activities with EC<sub>50</sub> of 13.75, 26.98 and 15.78 mg/mL, respectively [17]. It has been reported that the EC<sub>50</sub> of hot water extract of *Hypsizygus marmoreus* was 4.19 mg/mL [19], while the white mutant strain of the same species was less effective with an EC<sub>50</sub> of 18.85 mg/mL. In total, hot water extract of the mushroom tested by the DPPH method showed a moderate scavenging ability in relation to the other edible mushrooms researched.

There is a few studies about the addition of mushrooms in meat products. Pil-Nam *et al.* [26] proved that the adding of shiitake improved the sensory quality of frankfurters and slowed the lipid oxidation and aerobic bacteria growth during storage. In addition, Van Ba *et al.* [27] examined the effect of addition of shiitake (*Lentinula edodes*) extract on the quality characteristics of fermented sausages and decided that the addition of mushroom reduced lipid oxidation and retarded the growth of spoilage bacteria, as well as controlled the growth of pathogens. Also, Alnoumani *et al.* [28] showed remarkable inhibition of formation of lipid oxidation compounds when dried *Agaricus bisporus* powder was added to ground beef.

#### 4. Conclusion

Future research is needed with other types of mushrooms and meat products in order to come with a general conclusion about the feasibility of their application in meat products in general.

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# A novel fat modification strategy in fermented sausages by incorporation of gelled emulsions with fig seed oil

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**Abstract.** Gelled emulsion (GE) systems are one of the novel proposals for reformulation of meat products with healthier profiles. In this study, the quality of fermented sausages formulated with fig oil seed-in-water GE as partial or total beef fat replacers were studied. Control sausages (C) consisted of 100% beef fat, whereas GE treatments were formulated by replacing 50% (G1) or 100% (G2) of beef fat. Total replacement of beef fat with GE (G2 sausages) did not change 2-Thiobarbituric acid reactive substance (TBARS) levels in the sausages, whilst G1 sausages containing 50% GE had the highest TBARS levels among the sausages. In general, TBARS levels tended to increase at the end of the storage. The highest total carbonyl content was measured in C sausages with 100% beef fat; however, the final carbonyl contents of the sausages were not different. All sensory parameters were within acceptable ranges; color scores were higher in G2 sausages than in C sausages, and the rest of the sensory characteristics were similar to each other. Finally, it was concluded that utilization of gelled emulsion systems consisting of fig seed oil GE as beef fat replacers has good potential to enhance the chemical and sensory quality of fermented sausages.

## 1. Introduction

In our modern world, consumers mostly associate meat with a negative image as a high-fat and unhealthy food, and thus, an important goal for the meat industry is to suggest novel fat modification strategies to satisfy consumer needs. In this regard, today, an important goal for the meat industry is to develop novel lipid modification strategies. Incorporation of gelled emulsion (GE) systems in meat product formulations could be counted as one of the novel approaches in lipid modification that ensures both suitable product yield and healthier composition. A GE is defined as “an emulsion with a gel-like network structure and solid-like mechanical properties” [1]. Although oil-in-water emulsions have been widely used for lipid modification, GEs could be a better option to mimic functional and sensory characteristics of the animal fat used in most of the currently consumed meat products [2]. Although some studies have indicated that utilization of gelled emulsions had positive effects on emulsified meat products [2-5], the impacts of GEs in the formulation of fermented meat products have not been yet extensively studied.

Fig seed oil is a healthy fruit oil that highly contains oleic, linoleic, and linolenic fatty acids and is produced from an important exotic fruit, fig, which is a good source of dietary fibers, minerals, and



polyphenols [6]. In this study, we aimed to investigate the effects of fig seed oil-in-water GE systems as beef fat replacers on oxidative and sensory quality of fermented Turkish sausages (*sucuk*).

## 2. Materials and Methods

Fresh boneless post-rigor beef (*M. semitendinosus*), beef fat, and other ingredients were purchased from a local market in Izmir, Turkey. Cold-pressed fig seed oil was kindly donated by Egesia Co. (Aydin, Turkey). GE system was prepared using the method of Pintado *et al.* [3] with modifications. Fig seed oil (52.5 g/100 g emulsion) was emulsified with the aqueous phase (47.5 g/100 g emulsion) containing 41.8 g water/100 g emulsion, 5 g egg white powder/100 g emulsion, and 0.7 g microbial transglutaminase/100 g emulsion. After the emulsification process, the emulsion was cooled to room temperature and then cold-set for 12 h at 4Δ.

In the experimental design, each sausage treatment was adjusted to an initial total lipid content of 20%. Control (C) sausages consisted of 100% beef fat, whereas GE treatments were formulated by replacing 50% (G1) or 100% (G2) of the beef fat. Beef muscles and beef fat were separately minced through a 3 mm plate grinder (Arnica, Turkey). All ingredients (salt, saccharose, sodium nitrite, ascorbic acid, starter culture, cumin, garlic powder, sweet red pepper, and black pepper) were added to the treatments and homogeneously mixed. After stuffing into natural casings, the sausages were held at 22.5Δ and 60% relative humidity (RH) for 3 h and 23Δ and 88% RH until the pH reached 5.4 in a fermentation chamber (Wisd, South Korea). After fermentation, the sausages were ripened at 20-21Δ and 80-85% RH until the moisture content reached 40%.

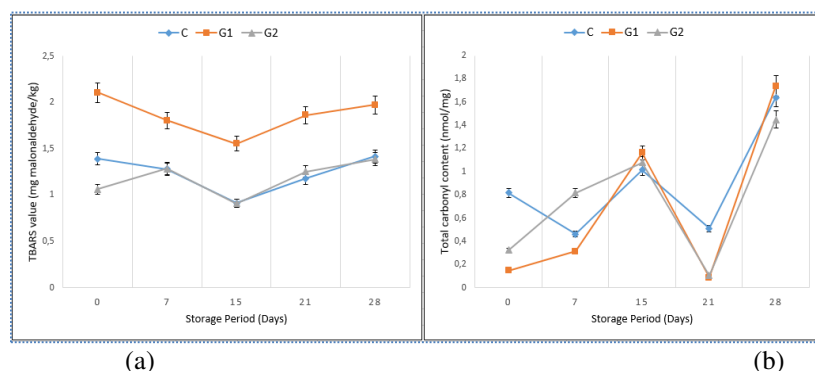
Oxidative stability of the sausages was analyzed by determining 2-Thiobarbituric acid reactive substances (TBARS) value [7] and total carbonyl content [8] throughout the 28 days of storage at 4°C. A sensory panel was carried out by 25 members using a 9-point hedonic scale (ranging from “like extremely” as 9 to “dislike extremely” as 1) to assess appearance, color, texture, flavor and overall acceptability of the sausage groups. Data were analyzed by One-Way Analysis of Variance (ANOVA) and Duncan’s Post-Hoc tests using the SPSS software for Windows (IBM, USA).

## 3. Results and Discussion

Lipid oxidation is one of the main causes for physical, sensory and nutritional quality deterioration in muscle foods. Since fermented meat products have relatively high lipid content, oxidation reactions could lead to changes in their chemical and/or sensory qualities. Analysis of TBARS is the most common method to determine the malonaldehyde concentration of meat products. TBARS values of the sausage groups are presented in Figure 1a. Initial TBARS values were between 1.064-2.104 mg malonaldehyde/kg sausage, indicating that oxidation reactions could occur during the fermentation and ripening periods. The results throughout the storage period showed that 100% replacement of beef fat with GE did not significantly change the oxidative stability of the sausages. Meanwhile, G1 sausages containing 50% GE had the highest TBARS values among the sausages during storage ( $P<0.05$ ). This result showed that the amount of GE incorporated into the formulation did not have a decisive impact on lipid oxidation. Lower TBARS values in G2 sausages could arise from the more protective effect of GE against oxidation when incorporated solely, compared to incorporation as a mixture of beef fat and GE (G1 sausages). In general, TBARS values of the sausages tend to increase at the end of the storage due to the propagation of lipid oxidation secondary products ( $P<0.05$ ). Wang *et al.* [9] reported that the replacement of pork back-fat with camellia oil gels decreased TBARS values of Harbin sausages significantly. In another study, it was found that lipid oxidation parameters showed higher susceptibility to oxidation in fermented sausages formulated with inulin linseed oil GE compared to conventional sausages [9]. Those results implied that the composition and characteristics of the unsaturated lipids in GE systems could be a determinative factor for oxidation level.

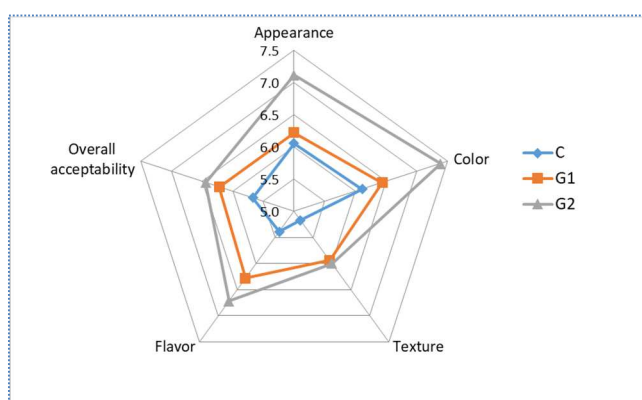
In recent years, protein oxidation of muscle foods has become a trending topic since the alterations in the proteins could cause many types of quality problems. One of the most noticeable modifications in oxidized food proteins has been highlighted as the generation of carbonyl compounds [11]. Initial carbonyl contents of the sausages (Figure 1b) were between 0.147-0.817 nmol/mg protein, and the

highest carbonyl contents were found in C sausages with 100% beef fat ( $P<0.05$ ). This data indicates that protein oxidation reactions in GE sausages could proceed more slowly during fermentation and ripening of the sausages. Although some fluctuations were observed in total carbonyl content of the sausages during storage, carbonyl contents mostly increased after 21 days ( $P<0.05$ ). Nevertheless, the final carbonyl contents of the sausages were not statistically different. Overall results indicated that incorporation of GEs with fig seed oil showed promising impact on retarding lipid and protein oxidation during cold storage of fermented sausages.



**Figure 1.** (a) TBARS values (mg malonaldehyde/kg sausage) (b) Total carbonyl content (nmol/mg protein) of the sausages during storage. C: control sausages formulated with 100% beef fat as lipid phase, G1: sausages formulated with 50% beef fat + 50% GE as lipid phase, G2: sausages formulated with 100% GE as lipid phase.

Since animal fat is a crucial ingredient that effects flavor, mouthfeel, and texture of the meat products, modification of lipid composition could greatly alter the sensory characteristics. Therefore, assessment of sensory quality is necessary for products where animal fat is replaced with alternative lipid sources. Sensory scores of the fermented sausages are presented in Figure 2. Appearance, color, texture, flavor and overall acceptability of the sausages ranged between 6.10-7.11, 6.11-7.39, 5.17-6.00, 5.39-6.72, and 5.67-6.44, respectively. The results showed that all sensory parameters were within acceptable ranges. Color scores were higher in G2 sausages than in C sausages ( $P<0.05$ ), indicating that panelists preferred the sausages formulated with 100% GE in terms of visual appearance. The rest of the sensory features were statistically similar to each other. The data is a good indicator of the promising effects of fig seed oil GE systems on sensory quality. In a similar study, it was reported that sensorial aspects of reduced-fat beef patties with microalgal oil gelled emulsions were similar to control patties with pork back fat [12]. Concordantly, Glisic *et al.* [10] found that fermented sausages formulated with inulin linseed oil GE were acceptable regarding all sensory attributes.



**Figure 2.** Sensory scores of fermented sausages. C: control sausage formulated with 100% beef fat as lipid phase, G1: sausages formulated with 50% beef fat + 50% GE as lipid phase, G2: sausages formulated with 100% GE as lipid phase.

#### 4. Conclusion

The present study indicates that fig seed oil GE systems in fermented sausages present favorable impacts in terms of oxidative stability and sensory scores. Utilization of this GE system showed that total fat replacement might be possible, since the GE system was able to maintain lipid and protein oxidation in the sausages at desired levels. Moreover, 100% GE sausages had better sensory scores than control sausages and sausages containing a mixture of GE and beef fat. Consequently, these fig seed oil GE systems could be used as novel animal fat replacers to develop healthier meat product formulations without altering chemical and sensory quality.

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## Physico-chemical parameters and acceptability and of spleen-treated beef patties

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**Abstract.** Iron deficiency is one of the world's most common disorders and it occurs when the amount of iron available is insufficient to meet an individual's needs. Spleen is known as a food product rich in iron content, and is a cheap offal. Therefore, consumption of spleen, both directly and indirectly, and especially for the treatment of anemia (iron deficiency) disorder is advised by the medical profession. However, consumption of cooked spleen is unacceptable to many people, due to its bloody structure. In this study, the effect of adding spleen at 0, 5, 10 or 15% to beef patties was studied and physico-chemical (pH, color and iron content) and sensory changes (color, odor, chewiness, flavor and overall acceptability) in the patties were investigated. Along with incremental increases of spleen content in beef patties, pH and iron content were increased, lightness L\* and redness a\* values were decreased, but yellowness b\* values were not significantly different between the patties with added spleen ( $P>0.05$ ). In terms of sensory analysis, panelists generally appreciated the patties with 10% spleen more than the other spleen levels.

### 1. Introduction

Although iron is an abundant element in the world, iron deficiency (anemia) is one of the most common human disorders worldwide. Iron deficiency occurs when the amount of iron available is insufficient to meet an individual's needs. Estimates indicate that over 2 billion people suffer from iron deficiency, and more than half of them are anemic. The prevalence of anemia is especially common among pregnant, infants, and children under the age of 2 years [1, 2]. Consequently, various treatments exist for iron deficiency, such as drug supplementation with iron, consumption of foods rich in iron (liver, spleen, fish, egg etc.) and products enriched with iron. For example, 100 g of raw beef spleen contains approximately 19 mg iron, and this amount increased up to 47.15 mg per 100 g after cooking [3]. Compared to other products (egg, fish etc.), although spleen is fairly rich in iron, it has some negative sensory properties such as texture, odor and flavor. Therefore, it is not an offal type that is frequently preferred by consumers. The aim of this study was to determine the effect of adding spleen to beef patty formulations on some physico-chemical (pH, color, iron content) and sensory properties of beef patties.



## 2. Materials and Methods

### 2.1. Preparation of beef patties

Fresh beef brisket and rib meat was obtained from a local meat processor in Denizli. Approximately, 2 kg of fresh meat was minced using a meat mincer (PM-70, Mainca, İspanya) through a plate with 3 mm holes. The minced beef mixture was divided into four to prepare the following formulations: Control (without spleen) and 5, 10 and 15% added spleen. For patty preparation, minced meat was mixed with salt (1%) and spleen, and then kneaded by hand for 15 minutes. Patties ( $25 \pm 1$  g) were molded using a metal shaper (6 cm diameter and 1 cm thickness) and polystyrene foam plate and stored at 2°C until analysis.

### 2.2. Analysis

The color values ( $L^*$  (lightness),  $a^*$  (redness),  $b^*$  (yellowness)) of patties was assessed with a colorimeter (Hunterlab Miniscan XE Plus, USA). To measure pH, 10 g of patty was homogenized with 90 ml of distilled water and homogenate pH was measured with a digital pH meter (Crison Basic 20, Spain). Before pH measurements, the pH meter was standardized using pH 4, 7 and 10 buffer solutions (Merck, Germany).

A Perkin-Elmer Analyst 700 atomic absorption spectrometer (AAS) (Norwalk, CT, USA) was used for analyses of the iron (Fe) in this work. The measurements were conducted in an air/acetylene flame. The running parameters for iron element were operated as suggested by the manufacturer. All measurements were performed in triplicate. Patty (1.0 g) was weighed on an analytical balance, and then 10 mL  $\text{HNO}_3$  was added. This mixture was predigested by standing in open vessels for a minimum of 15 mins before sealing the vessels. Digestion was conducted using a microwave system, power set at 1030 and 1800 watts, ramp time 20-25 min, hold 15 mins. Preliminary experiments showed that 15 min hold digestion time was suitable for digests without insoluble materials and at 200°C.

The patties were evaluated by a 20-member semi-trained panelist team selected from Pamukkale University Department of Food Engineering students. The patties were cooked in a conventional oven (Termikel 13007, Turkey) at 130°C for 20 min until the internal temperature reached 80°C and then, all cooked patties were coded with 3-digit random codes and offered to the panelists in a random order. Sensorial properties color, odor, chewiness, flavor and overall acceptability were evaluated using a seven point hedonic scale, ranging from dislike extremely unacceptable (score: 1) to like extremely acceptable (score: 7).

The statistical design of the study was 4 (treatments) \* 3 (replications) randomized block design and all parameters were measured in duplicate ( $n = 24$ ). A one-way analysis of variance (ANOVA) and Duncan's Multiple Range Test were performed to analyze in order to evaluate effects on the treatments and the storage periods using SPSS for Windows (SPSS version 15,0 for Windows). Critical difference was determined at the 5% significance level.

## 3. Results and Discussion

Color plays an important role in both the quality and consumers' acceptance of meat and meat products. Physico-chemical properties (color, pH and iron content) of beef patties are presented in Table 1. Patties containing spleen had lower  $L^*$  (lightness) values than control patties ( $P < 0.05$ ). A statistical difference in  $a^*$  (redness) values among the patties was observed, shown in Table 1. As expected,  $L^*$  (lightness) and  $a^*$  (redness) values decreased with the increasing amounts of spleen due to the fact that spleen has a substantially red pigment. The patties containing 15% spleen had lowest  $L^*$  and  $a^*$  values among the patties ( $P < 0.05$ ). The addition of spleen did not significantly alter the  $b^*$  (yellowness) values ( $P > 0.05$ ), but the  $b^*$  values of the patties fluctuated.

The pH of the patties increased from 6.50 to 6.90 as the proportion of spleen increased from 0 to 15% in the beef formulation. Patties with spleen had slightly higher pH than the control patties ( $P < 0.05$ ).

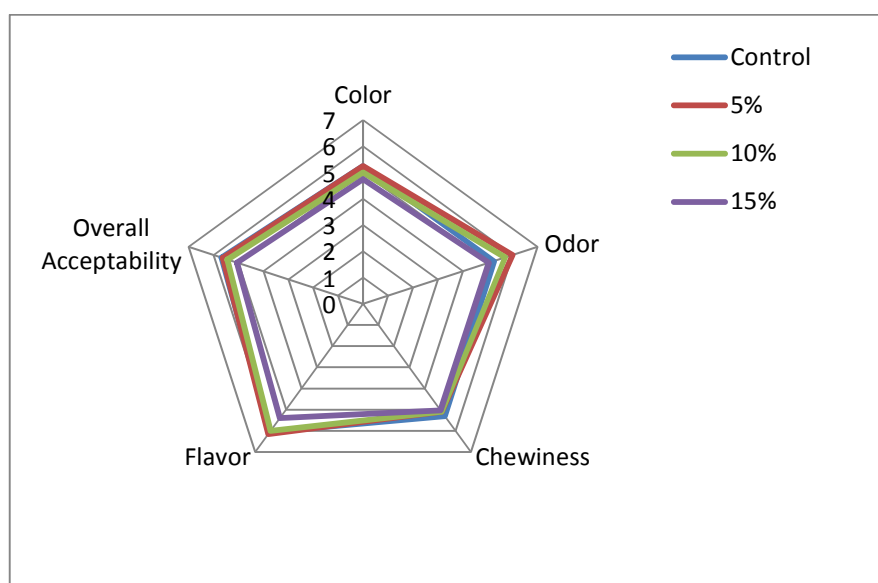
Iron contents of the patties were between 11.8-28.5  $\mu\text{g/g}$ . The iron content of patties with 15% spleen was approximately 2.5-fold higher than that of control patties. Moreover, as the proportion of spleen increased in the beef patty formulations, iron content increased and differences were statistically significant ( $P < 0.05$ ).

**Table 1.** Physico-chemical properties (color, pH and iron content) of beef patties

	L* (lightness)	a* (redness)	b* (yellowness)	pH	Iron Content ( $\mu\text{g/g}$ )
C	41.31 $\pm$ 0.23 <sup>a</sup>	11.90 $\pm$ 0.41 <sup>a</sup>	21.59 $\pm$ 0.29 <sup>b</sup>	6.50 $\pm$ 0.02 <sup>b</sup>	11.8 $\pm$ 0.4 <sup>d</sup>
5%	36.99 $\pm$ 0.31 <sup>b</sup>	7.05 $\pm$ 0.40 <sup>b</sup>	23.96 $\pm$ 0.21 <sup>a</sup>	6.87 $\pm$ 0.01 <sup>a</sup>	15.5 $\pm$ 0.7 <sup>c</sup>
10%	34.84 $\pm$ 0.19 <sup>c</sup>	6.12 $\pm$ 0.35 <sup>bc</sup>	22.99 $\pm$ 0.27 <sup>a</sup>	6.88 $\pm$ 0.01 <sup>a</sup>	23.2 $\pm$ 1.2 <sup>b</sup>
15%	32.45 $\pm$ 0.32 <sup>d</sup>	6.01 $\pm$ 0.33 <sup>c</sup>	23.01 $\pm$ 0.31 <sup>a</sup>	6.90 $\pm$ 0.02 <sup>a</sup>	28.5 $\pm$ 1.3 <sup>a</sup>

a, b, c, d Values with different superscripts within the same column are significantly different ( $P < 0.05$ ); C: control beef patties without spleen; 5%: beef patties with 5% spleen, 10%: beef patties with 10% spleen; 15%: beef patties with 15% spleen

Results of sensory analysis (color, odor, chewiness, flavor and overall acceptability) of beef patties are given in Table 2. Patties with 15% spleen were a different color to control patties ( $P < 0.05$ ), while color differences between control and patties with 5% spleen were not significant ( $P > 0.05$ ). Although patties with 5% spleen had the highest sensory odor scores (6.00), there was no odor difference between patties with 5% and 10% spleen ( $P > 0.05$ ). Chewiness scores were not different between the patties (Table 2) ( $P > 0.05$ ). The beef patties with 15% spleen had a significantly different flavor than beef patties prepared with less spleen or without spleen. The overall acceptability scores of the patties with 5% spleen and control patties were similar ( $P > 0.05$ ). Control patties were the most acceptable, overall, while patties with 15% spleen had the lowest overall acceptability (5.68 $\pm$ 0.24 and 5.06 $\pm$ 0.30, respectively). Addition of 5% spleen did not produce a negative impact on sensory properties. However, with the higher percentages of spleen in the patty formulations resulted in lower sensory scores of the patties, except that of odor. Krishnan and Sharma (1990) reported that offal (rumen and heart meat in equal proportions) in buffalo meat sausages did not produce any negative effect on sensory properties (appearances, color, flavor, juiciness and overall acceptability).



Control: beef patties without spleen; 5%: beef patties with 5% spleen, 10%: beef patties with 10% spleen; 15%: beef patties with 15% spleen

**Figure 1.** Sensory properties (color, odor, chewiness, flavor and overall acceptability) of beef patties.

#### 4. Conclusion

Spleen is a food (along with liver) recommended by the medical profession for the treatment of iron deficiency anemia. The development of beef patties fortified with the spleen could help older adults, pregnant women and infants achieve their targeted iron requirements, thus reducing the risk of anemia. Our study on the physico-chemical properties of the patties with spleen showed that increasing the percentage of spleen incorporated in the beef patties does not affect b\* (yellowness) or pH, while the L\* (lightness) and a\* (redness) values decrease, but iron content increases. The findings from this research showed that beef patties with 5% spleen and control patties (no spleen) were similarly favorably assessed in terms of sensory scores. Spleen has potential to be used to successfully enrich beef patties with iron, providing a new and healthier product.

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## Fatty acid composition of *Acipenseridae* – sturgeon fish

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**Abstract.** Fish meat is considered to have a beneficial nutritional composition and a favorable effect on human health. Fish meat is a significant source of highly unsaturated fatty acids with beneficial effects on health of consumers. Therefore, knowledge of the fat, protein and especially quality of lipids in fish is very important. The objective of this summary is to synthesize data on the fatty acid composition of different sturgeon species, the meats of which could become more common in Serbia. Sturgeon, due to their favorable sensory properties, are of increasing interest to consumers. These fish are also interesting to fish farms because they are relatively easy to breed, grow fast, and are relatively resistant to diseases. Quality parameters of sturgeon meat are not well studied and there are few data to date.

### 1. Introduction

Siberian sturgeon and other sturgeon species are food fish of commercial significance in many countries. Recently, it has been recognized that sturgeon farming seems promising due to the fish's excellent meat quality and relatively high market prices. Sturgeon are increasingly consumed and their meat is well accepted by consumers due to their sensory properties, high concentrations of unsaturated fatty acids and other nutritional properties. Consumption of sturgeon meat has increased recently. According to Gisbert and Williot [1] sturgeon are suitable for aquaculture due to the fish's relatively good acceptance of formulated feed, rapid growth rate and adaptation to rearing systems. The quality of reared fish is influenced by several factors including nutrition, genetics, water quality, environmental conditions, health conditions, and management practices on the fish farm. The new technologies of sturgeon breeding are mainly based on intensive culture systems and have enabled increased amounts of sturgeon meat to be released on the EU and international markets in recent decades [2].

Sturgeon meat is appreciated by consumers due to its sensory properties and nutritive value. The meat has high contents of highly unsaturated fatty acids (HUFA), which could play an important role in protection of humans from cardiovascular diseases. Essential polyunsaturated fatty acids (PUFA) are not synthesized in the human body but are efficiently synthesized by some aquatic organisms; therefore, a good source of essential PUFA is marine and freshwater fish [3,4]. Docosahexanoic acid (DHA) and eicosapentaenoic acid (EPA) have a positive effects in preventing hypertension and cardiovascular diseases and also have beneficial effects by improving the immunological system [5]. The fatty acid composition of the lipids in sturgeon meat has been relatively little studied in comparison with other fish species. However, knowledge of the fatty acid composition of sturgeon meat is required



to properly assess the value of these fish. The objective of this summary was to synthesize data on the fatty acid composition of sturgeon meat, and compare the content of fatty acids in the meat of different sturgeon species, some of which could become more common in Serbia.

## 2. The effect of sturgeon species on chemical composition and fatty acid profile

Sturgeon is a common name of 27 species of fish belonging to the family *Acipenseridae*. The chemical and fatty acid composition of muscle tissue in some sturgeon species and hybrids (Russian sturgeon (*Acipenser gueldenstaedtii*), Siberian sturgeon (*Acipenser baerii*), hybrid (*Acipenser baerii* Br × *Acipenser medirostris* Ayres) [6-8] have been examined so far. The chemical composition (Table 1) and percentages of fatty acids (Table 2) were highly variable among the different sturgeon species but also within the same species [9].

Table 1 shows the varied contents of lipids and water in all studies. A negative correlation between lipid and water contents in sturgeon meat was observed in all studies, which is in agreement with the results obtained for other fish species [10]. The lipid content in the meat of different cultured sturgeon species varied, and reported percentages ranged from 5 to 15% [11].

The water content of wild sterlet examined by Ljubojevic et al. [10] was slightly lower (75.38 g/100g vs 77.5-77.2), protein content was higher (17.54 g/100g vs 13.1-13.8), and lipid content was within the same range 4.8-6.1 g/100 g as was reported [12] for cultured sterlet. Significant differences in the content of ash in examined sturgeon meats (Table 1) could be due to the presence of small bones in fish fillets. Namely, the calcium, released during bone demineralization, can contribute to a greater mass fraction of ash in the total chemical composition of fish meat. Sturgeon are carnivores and require a high protein feed for optimal health.

**Table 1.** Proximate composition of different sturgeon species

Proximate composition	Stellate sturgeon	Beluga sturgeon	Sterlet ( <i>Acipenser ruthenus</i> )	Russian sturgeon ( <i>Acipenser gueldenstaedtii</i> ), juveniles	Hybrid (SSZ) Siberian sturgeon × (Siberian sturgeon × green sturgeon) – <i>Acipenser baeri</i> Br × ( <i>Acipenser medirostris</i> Ayres)
Fish weight g	652.5	201.5	1320 g	27.23±0.98 g	1 527± 158
Moisture %	77.8	83.0	75.38±0.35	78.24±0.05	73.52±0.55
Proteins %	16.7	14.9	17.54±0.23	10.71±0.04	16.51±0.38
Fat %	3.9	1.1	5.39±0.14	6.05±0.07	8.9±0.5
Ash%	1.6	1.0	0.93±0.09	2.36±0.02	0.98±0.05
Cholesterol content, mg/100g			73.59±0.11a		
Reference	Paltenea et al. [14]	Paltenea et al. [14]	Ljubojević et al. [10]	Sener et al. [6]	Jankowska et al. [8]

**Table 2.** Fatty acid profiles of different sturgeon species

Fatty acids	Wild Stelet sterlet ( <i>Acipenser ruthenus</i> )	The farmed Siberian sturgeon ( <i>Acipenser baerii</i> )	Reciprocal hybrid (SSZ) Siberian sturgeon × (Siberian sturgeon × green sturgeon) – <i>Acipenser baeri</i> Br × ( <i>Acipenser baeri</i> × <i>Acipenser medirostris</i> Ayres)	Russian sturgeon ( <i>Acipenser gueldenstaedtii</i> ), juveniles
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C14:0	3.31±0.27b	4.08±0.187	4.47±0.36	4.14±0.1
C15:0	0.66±0.05ab			
C16:0	25.09±0.14b	19.2±0.271	19.79±1.92	
C16:1	13.27±0.12	0.352±0.012	8.18±0.68	
		6.24±0.121		
C17:0	1.09±0.14			
C18:0	2.38±0.12e	1.91±0.104	1.91±0.15	2.68±0.1
C18:1 cis-9	24.87±0.15	25.1±0.47	27.77±1.48	21.57±0.5
C18:1 cis-11	7.05±0.16		2.80±0.14	
C18:2, n6	2.8±0.19	7.59±0.301		11.24±0.3
C18:3, n6	0.69±0.51	0.410±0.013	5.63a±0.19	0.29±0.1
C18:3, n3	4.34±0.1	1.49±0.059	1.22a±0.88	1.48±0.2
C20:0	0.14±0.04			0.13±0.1
C20:1	0.77±0.11	1.50±0.063	3.97±0.25	1.60±0.2
C20:2	0.47 ±0.07	0.421±0.013		
C20:3, n6	0.2±0.11			
C20:3, n3	0.55±0.05			0.24±0.1
C20:4, n6	1.54±0.08	0.898±0.050	0.63a±0.07	0.86±0.1
C20:5, n3	4.93±0.09	6.83±0.129	5.46a±0.38	6.43±0.3
C22:5, n3	2.86±0.04	1.84±0.024	1.45a±0.07	
C22:6, n3	3.79±0.09	7.95±0.343 b,	9.34a±0.72	11.06±0.9
SFA	32.67±0.34	27.0±0.226 a,	26.71±1.92	
MUFA	45.97±0.15	39.9±0.459	46.21a±1.27	23.29±0.8
PUFA	22.17±0.37	32.9±0.382	27.08a±1.68	
n-3	16.47±0.05	20.2±0.354 b,c	19.58b±0.68	19.21±0.8
n-6	5.7±0.36	9.99±0.263	6.59a±0.12	12.39±0.4
N3/n6	2.9±0.19a	2.04±0.076 b,	2.97a±0.11	
N6/n3	0.35±0.02			
PUFA/SFA	0.68±0.01			
USFA/SFA	2.09±0.03	2.70±0.031		
Reference	Ljubojevic et al [10]	Nieminen et al. [7]	Jankowska et al. [8]	Sener et al. [6]

Data are means±S.E.M. (n = 8). Different superscripts within the same rows differ (P < 0.01), USFA – unsaturated fatty acids, SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids).

### 3. The effect of nutrition on chemical composition and fatty acid profile of sturgeon

Bieniarz et al. [13] found freshwater carnivorous fish can be characterized by higher n-3/n-6 fatty acid ratios than phytophagous and benthophagous fish. The fatty acid composition of muscle lipids in fish is greatly influenced by dietary fatty acids, and linear correlations exist between individual fatty acids in muscle tissue, total lipids and their concentrations in dietary lipid. Nieminen et al. [7] reported the fatty acid profile of the diet diverged from the fatty acid profile of the tissues, where the sturgeons accumulated particular HUFA. According to Sener et al. [6], whole body lipid contents in juvenile Russian sturgeon (*Acipenser gueldenstaedtii*) fed on feeds including fish oil, soybean oil and sunflower oil were 4.65%, 4.73% and 5.19%, respectively. The total n-3 and n-6 fatty acids (in the total fatty acid content) of the fish differed significantly, as the percentage of total n-6 fatty acids was higher (22.58% and 22.98%) in fish fed vegetable oils than in the fish oil group (11.39%), while the percentage of n-3 fatty acids was higher (21.57%) in the group fed fish oil than that in the vegetable oil groups (13.15% and 15.00%). The content of DHA was higher in fish fed a diet containing soybean and sunflower oil, and similar findings were reported for white sturgeon (*Acipenser transmontanus* R.) [15]. Russian sturgeon (*Acipenser gueldenstaedtii*) [16, 17], juvenile Iranian sturgeon (*Acipenser persicus*) [18] and juvenile Beluga sturgeon (*Huso huso*) [19] showed the selectivity and requirement of DHA in sturgeon species. Generally, sturgeon fed diets supplemented with vegetable oils accumulated n-3 PUFA (EPA and DHA). Furthermore, sturgeon species are able to elongate linoleic acid and  $\alpha$ -linolenic acid to arachidonic acid (AA), EPA and DHA [6, 18, 19]. Sturgeon species appear to require both n-3 and n-6 fatty acids in their nutrition, and accumulation of these fatty acids in the meat was affected by the fatty

acids in fish diets. Knowledge of the cholesterol content in food is also very important, especially in fish meat, consumption of which is increasing based on the recommendations of healthy nutrition. Kopicova and Vavreinova [20] reported total cholesterol in starlet as being 61 mg/100g.

#### 4. Recommended ratio of n-6/n-3 essential fatty acids

Fish meat should be included in human nutrition for at least three reasons: as a general source of nutritional components; as low fat, high protein food; and as a source of polyunsaturated fatty acids. The recommended ratio of PUFA to saturated fatty acids (PUFA/SFA) should be increased to >0.4 [21]. Wood et al. [22] reported that all examined fish species have favorable (from 0.63 to 0.92) PUFA/SFA ratios. The n-6/n-3 ratio in all examined fishes was in the optimal range of 2.0-4.0 for human health as suggested by Pepping [23]. From the data shown in Table 2, n-6/n-3 ratios of different sturgeon species were <4.0, which is in accordance with the recommendation of Simopoulos [24] for human nutrition. Moreover, the European Food Safety Authority recommends daily intake amounts of 250 mg of EPA and DHA [25]. Meat of terrestrial farm animals has been implicated as a main cause of the imbalanced fatty acid intake of today's consumers, due to the fact that some meats naturally have a PUFA/SFA ratio of around 0.1 [22]. Sturgeon lipids are particularly rich of polyunsaturated fatty acids (PUFA) that are only slowly synthesized in humans, which is the major difference between fish meat and meat of farmed terrestrial animals [26]. Knowledge of the chemical and fatty acid composition of freshwater fish including sturgeon is important to nutritionists, who are interested in finding sources of low fat, high protein food with desirable fatty acid composition and favorable amounts of total cholesterol. Sturgeon meat contains biologically active protein that is characterized by a good composition of amino acids, HUFAs including EPA and DHA, and fat-soluble vitamins. Additionally, sturgeon meat is a good source of micro- and macro-elements [27, 28].

#### 5. Conclusion

In conclusion, all sturgeon species likely have potential to become a desirable fish in terms of their fat content and composition. Higher contents of PUFA mostly originate from the feed, so appropriate nutrition of farmed sturgeon might be a solution for obtaining optimal fatty acid composition of sturgeon meat from the nutritional standpoint. The potential to exploit the presently insufficiently used sturgeon species as high-protein foods and to introduce new sturgeon species in Serbian aquaculture underscore the need for reliable analytical data.

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# Influence of different sources of fat on lipid index of muscle and fat tissue of pigs

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**Abstract:** Of the total meat production in Serbia, pork makes up more than one half. This meat is often associated with cardiovascular diseases due to its high contents of fat and saturated fatty acids. The aim of this study was to determine the effect of various sources of fat in pig feeds on lipid indices of fat and muscle tissue of pigs from the point of view of consumer health needs. A total of 30 Yorkshire × Landrace crossbred pigs were divided into three experimental groups of 10 individuals and fed a complete finisher mixture for fattening pigs, with standard raw materials and chemical composition but with differing sources of fat. The results obtained show fat sources in pig feed significantly influenced the lipid indices, and the differences were more pronounced in fat than in muscle tissue of pigs.

## 1. Introduction

Pig production is an important part not only of agricultural production but also of the agricultural service industries as suppliers of necessary equipment, raw materials, and preventive and protective materials [1]. The rapid development of pig breeding has led to an increase in the reproductive efficiency of sows and carcass quality, but the meat content of carcasses has almost reached a physiological maximum. The market requires quality meat with a favorable meat:fat ratio, so the goal of each meat industry is based on the production of meat that fulfills quality criteria. Production and consumption are in a causal relationship, and so high consumption causes high production and *vice versa*. The same relationship exists between demand and price, with a low price, of course, increasing consumption [2].

Pigs have a short reproductive cycle that lasts about six months. In intensive sowing, during the production cycle, in one year from one sow, 36 fattening animals can be produced, of which about 1500 kg of meat can be obtained. Pork is accepted as consumable by many cultural groups and is often further processed. In the culinary industry, it is more widely used than other meat species, while beef production has been suppressed, as its market price is lower. The total production of pigs is unevenly distributed worldwide and very differently across the continents, which is conditioned by a number of factors, such as climatic conditions, surface, structure and quality of arable land, development of agro-industrial complex and the like. The continent that stands out for pig production is Asia.

Pig production in Serbia is the most important branch of animal production, both from the socio-economic and from the biological and zootechnical points of view [3]. Pigs in Serbia are the most common meat animal, and production is characterized by the growing participation of large farms (capacity 10,000 to 20,000 pigs per year), while small producers are slowly disappearing. The quality



of pigs in Serbia has improved significantly and can be said to be slowly approaching the world standard. What permanently burdens the production of pigs and pork in the world, but is much more pronounced in Serbia, are the large fluctuations in the price of live pigs. This is the result of major disorders in the supply and demand of pigs, but also the enormous variation in the price of the basic pig feed, corn [1]. In 2019, there were almost 3 million pigs in Serbia. According to the Statistical Yearbook of Serbia, the annual production of pork is about 250,000 tons. In Serbia, pigs are the most commonly slaughtered animal, and therefore, this type of meat is the most common on the table of average consumers [4]. Consumption of pork in Serbia is at the level of the European average, around 43 kg per capita per year. Meat consumption has fallen to today's levels as a result of the decline in living standards over the past twenty years [1].

The basic task of raising livestock is achieving high production of quality meat with minimal food consumption and with as low as possible production costs. The needs of pigs for nutrients and energy are expressed as the need for life sustainability and production. The need for fat in pigs is considered to be relatively small, up to 2%. Lack of essential fatty acids can lead to severe disorders, and a lipid amount of up to 6% in the feed accelerates its utilization.

Fats have multiple relevancies in human nutrition, both for food acceptability and for application in the technological processes of food production. Their energy significance is based on the fact that one gram of fat has an energy value of 9 kcal (37 kJ), which is much more than the energy value (4 kcal per g (17 kJ)) of proteins and carbohydrates [5, 6]. In human diets, fat is particularly important as the carrier of fat soluble vitamins (A, D, E and K) and support for their absorption in the digestive tract. Human organs do not have the ability to synthesize two polyunsaturated fatty acids (PUFA), i.e., linoleic acid (LA; C18: 2 n-6) and alpha-linoleic acid (ALA; C18: 3 n-3). The lack of these essential fatty acids can cause disease in humans.

The attention of the professional and scientific communities today is focused on finding an adequate means of controlling cardiovascular diseases in humans. Research focuses on controlling the qualitative relationship between saturated and unsaturated fatty acids in fats. The class of n-3 PUFA is derived from ALA, the main source of which is fish oil, while the class of n-6 PUFA is derived from LA, which is mainly found in vegetable oils [7]. The desaturation and chain elongation of ALA and LA, by which their PUFA derivatives are formed, are catalyzed by the same enzyme (desaturase). This creates competition for this enzyme among these essential fatty acids, so increasing the concentration of LA can inhibit the conversion of ALA into its derivatives, and *vice versa*, intaking predominantly ALA and/or eicosapentaenoic acid can reduce the production of LA derivatives and arachidonic acid. These dis-balances can impair the ratio of n-6:n-3 fatty acids in the body [8, 9]. Research shows an important role is played by the interaction of these two groups of dietary PUFA (n-6 derived from LA and n-3 derived from ALA) in the development of cardiovascular diseases in humans. Contemporary human diets have undesirable ratios of n-6:n-3, often exceeding 25:1, although this ratio should be 4:1 [5, 10]. The nutritional value of fat and its importance for human health is most often expressed through the n-6:n-3 ratio, but recently, lipid indices have been used that are directly related to the fatty acid composition of the tissue. Calculating the atherogenic (AI) and thrombogenic index (TI) indicates the potential for cardiovascular disease, and for the purpose of protecting consumers against hypercholesterolemia, a condition that can cause atherosclerosis, the hypocholesterolemic/hypersterolemic index (h/H) is calculated.

As animal nutrition can affect the ratio of n-6:n-3 fatty acids in pigs, lipid indices can also be affected. The fatty acid profile of feeds is directly reflected in the fatty acid profile of the animal tissues [11]. The aim of this study was to determine the effect of various sources of fat in pig feeds on fatty acid composition and lipid indices of fatty and muscle tissue from the pigs, in terms of consumer health needs.

## 2. Materials and Methods

For the purposes of this study, 30 Yorkshire × Landrace crossbred pigs were used with a starting weight of 60 kg. The pigs were divided into three experimental groups of 10 individuals and fed with a complete finisher mixture for fattening pigs, with standard raw materials and chemical composition. The feed for the pig groups differed in that the experimental group E-I had sunflower seed in the feed, the second experimental group E-II had a linseed preparation at the recommended 2.5% level in the feed (Vitalan 85-15; Vitalac, France), and feed for the third experimental group E-III had soybean grits. Vitalan 85-15 contained 85% extruded linseed and 16% wheat bran.

After sacrificing the pigs, the fatty acid compositions of muscle and fat tissues were determined and used to calculate the lipid indices (AI, TI, h/H) according to the following formulas:

$$AI = [(C12:0) + (4 \times C14:0) + (C16:0)] / [\Sigma n6 + \Sigma n3 + \Sigma MUFA]$$

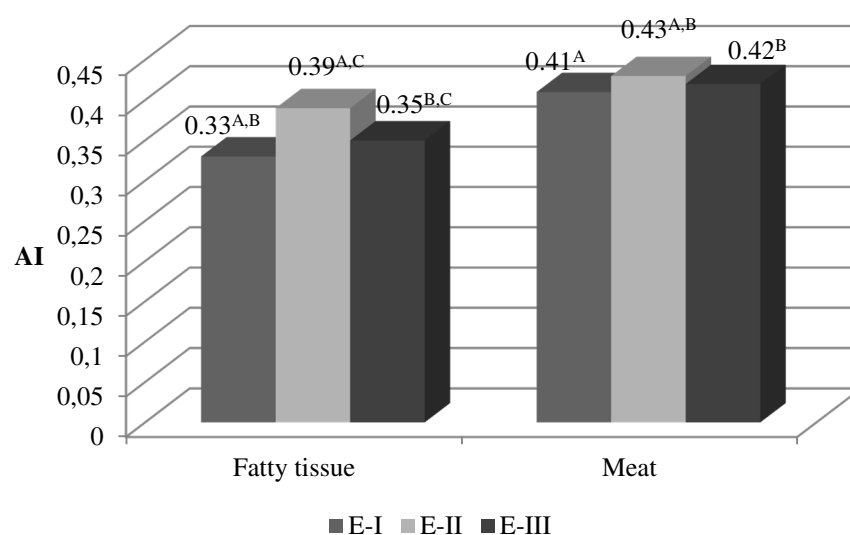
$$TI = [(C14:0) + (C16:0) + (C18:0)] / [0.5 \times \Sigma MUFA + (0.5 \times \Sigma n6) + (3 \times \Sigma n3) + (\Sigma n3 / \Sigma n6)]$$

$$h/H = [(C18:1 + C18:2 + C18:3 + C20:3 + C20:4 + C20:5 + C22:4 + C22:5 + C22:6) / (C14:0 + C16:0)]$$

Statistical data processing was done in GraphPad Prism software version 7.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). The results are presented graphically as intermediate values using Microsoft Office Excel 2010. Comparison of lipid indices among examined groups of pigs was done using one-factor analysis of variance (ANOVA). Statistical significance is shown at  $p < 0.05$ .

## 3. Results and Discussion

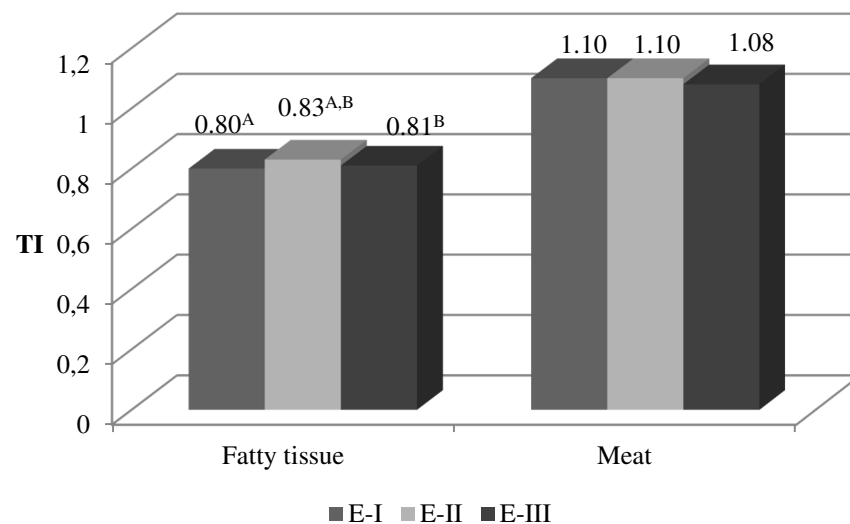
Figures 1 and 2 show AI and TI indices of fat and muscle tissue from pigs fed with different fat sources in feed. Statistically significant differences were found between the AI of fatty tissue of the examined groups of pigs ( $p < 0.05$ ). The smallest AI index was  $0.33 \pm 0.01$  in E-I, and the largest ( $0.39 \pm 0.01$ ) was in E-II pigs that were fed on linseed mixture. The AI of the meat was statistically significantly higher ( $p < 0.05$ ) in E-II ( $0.43 \pm 0.01$ ; fed with linseed mixture) than in E-I ( $0.41 \pm 0.01$ ) and E-III ( $0.41 \pm 0.01$ ) pigs.



**Figure 1.** Atherogenic indices (AIs) in fatty tissue and meat from pig groups fed on mixtures with sunflower (E-I), linseed (E-II) or soybean (E-III). Bars with the same letter A, B or C differ significantly ( $P < 0.05$ ).

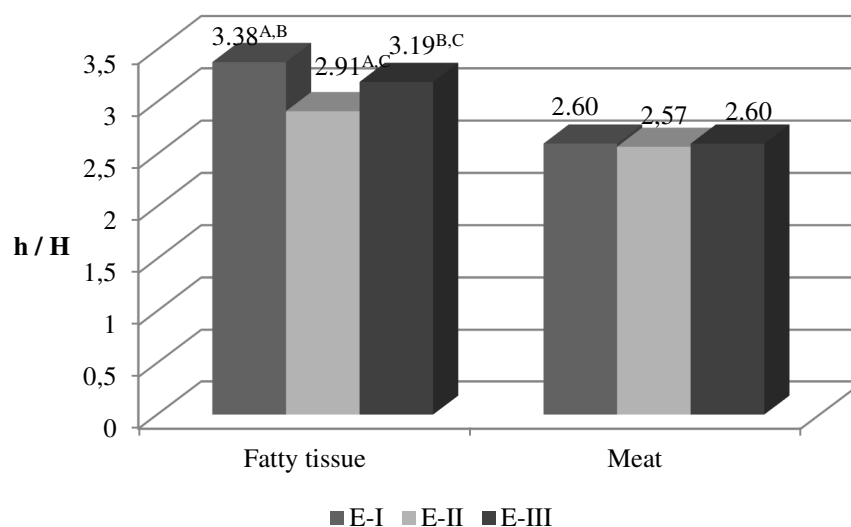


The average TI of fatty tissue from pigs fed with added linseed ( $0.83 \pm 0.02$ ) was statistically significantly higher ( $p < 0.05$ ) than the average TI of fatty tissue from E-I ( $0.81 \pm 0.01$ ) and E-III ( $0.81 \pm 0.01$ ) pigs (Figure 2). No statistically significant differences were found between the average TI of muscle tissue from the pig groups (TIs ranged from 1.08 to 1.10).



**Figure 2.** Thrombogenic indices (TIs) in fatty tissue and meat from pig groups fed on mixtures with sunflower (E-I), linseed (E-II) or soybean (E-III). Bars with the same letter A or B differ significantly ( $P < 0.05$ ).

A statistically significant difference ( $p < 0.05$ ) between the h/H index was found in the fat tissue of all three examined pig groups. This index ranged from  $2.91 \pm 0.04$  (E-II) to  $3.38 \pm 0.06$  (E-I). The h/H index of pig muscle tissue ranged from  $2.57 \pm 0.06$  (E-II) to  $2.60 \pm 0.03$  (E-I and E-III). The differences between the average values of h/H of muscle tissue from pigs fed with different fat sources were not statistically significant.



**Figure 3.** The ratio of hypocholesterolemic/hypercholesterolemic fatty acids (h/H) in fatty tissue and meat in from pig groups fed on mixtures with sunflower (E-I), linseed (E-II) or soybean (E-III). Bars with the same letter A, B or C differ significantly ( $P < 0.05$ ).

Small AI or TI indicates nutritionally more valuable food, or that the food could lower the risk of cardiovascular diseases associated with fat intake [12]. High h/H indices indicate the higher nutritive value of fat. Previous studies on this topic showed the use of linseed (i.e. flaxseed) in pig nutrition significantly affects the n-6:n-3 ratio. Thus, the ratio of n-6:n-3 in the muscle tissue from pigs fed with added linseed was 13.67, 32.40 and 17.84 (E-II, E-I and E-III pigs, respectively) [13]. The n-6:n-3 ratio in the fatty tissue from pigs feed with added linseed was 10.23, 27.30 and 17.74 (E-II, E-I and E-III pigs, respectively) [13]. This raises the question of whether lipid indices are more relevant indicators of the nutritional value of fat in food.

According to literature data, age and provenance in broilers does not significantly affect lipid indices of breast and thigh meat. The AI of breast meat was 0.46-0.54, TI was 0.99-1.10, and h/H index was 1.81-2.18. The same indices were 0.40-0.46 (AI), 0.96-1.08 (TI) and 2.22-2.57 in thigh meat [14]. In muscle tissue of pigs, AI was 0.47, TI was 1.06 and h/H ratio was 2.19, in pig fat tissue, AI was 0.48, TI was 1.14 and h/H ratio was 2.26, while in pig liver, AI was 0.30, TI was 0.94 and h/H ratio was 3.40. AI and TI in the liver have lower values than in fat and muscle tissue, while the h/H ratio is higher in liver, which is beneficial for consumer health [15]. Muscle tissue of different breeds of cattle had AI of 1.85, and 1.62, TI of 3.95 and 3.63 and h/H ratio of 0.52 and 0.62 [16]. In groups of sheep with different fat sources in their diets, AIs were 0.92 and 0.77, and TIs were 2.14 and 1.64 [17]. Fish AI was 0.43-0.46, TI was 0.32-0.36 and h/H index was 2.30-2.59, depending on the season [18]. Other low AI and TI and high h/H indices were also reported by other authors [19, 20].

#### 4. Conclusion

Different sources of fat in pig feed significantly affect the lipid indices, and these differences are more pronounced in fat than muscle tissue of the pig.

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# Fast, simple and reliable triglyceride composition analysis of milk fat for discrimination of cheese origin and adulteration detection

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**Abstract.** Optimization of a novel high performance liquid chromatography with refractive index detection method, together with accelerated solvent extraction for determining the triglyceride composition of lipids to enable rapid discrimination of goat and cow cheeses is presented. The method was applied to determine adulteration of goat and cow cheeses with vegetable oils/fats, to distinguish cheese analogues from natural cheeses, and to determine the amounts of palm fat added to cheese. Principal component analysis of triglycerides grouped by equivalent carbon number was used for confirmation of cheeses' animal origins. A total of 130 cheeses or cheese analogues were included in this study. The method was calibrated in the range of 0 to 50 % palm fat in milk fat, with recovery values being from 46.50 % to 108.48 %. The content of palm fat found in adulterated cheese was from 10 to 16 %, and 5 to 10 % in cow and goat cheeses, respectively. Due to the rapidity of the method, employment of relatively inexpensive equipment and the reliability of the results, the method proved to be suitable for analytical laboratories that are required to process large numbers of cheese samples in a short time.

## 1. Introduction

Cheese is an important source of essential substances and fundamental ingredients in diets that prevent nutritional deficiencies [1]. Among other cheese constituents, milk fat (MF) is one of the major components and can be present in amounts up to 40 % [2]. The main lipid constituents of MF are triacylglycerols (triglycerides, TGs), as approximately 98 % of MF consists of TGs [3]. Some fatty acids (FA) in the milk TGs are only found in ruminant MF [4,5].

The trends of substituting goat cheese with cow or natural cheeses with cheese analogues are substantially and clearly economically motivated. This kind of food fraud has not only a financial effect but also has impact on human health and nutrition. Lactic acid bacteria present in natural cheeses produce numerous beneficial effects in consumers [6], but cheese analogues lack these microorganisms. Long-term deprivation of nutrients normally provided by natural cheeses could have adverse effects on consumers' health if cheese analogues are unwittingly consumed [1,6].

Efforts to detect the animal origin of cheese or cheese adulteration and fraud have resulted in development of numerous analytical methods. Techniques like real-time polymerase chain reaction (PCR), electrophoresis, gas and liquid chromatography were employed for milk and cheese authenticity determination [7-10]. Since the cheese adulteration and frauds include partial or whole milk fat substitution with foreign fat, emphasis could be on development of reliable methods for



analysis of TGs and FAs found in MF. Determination of the lipid fraction of MF constituents included, for the most part, gas and liquid chromatography techniques [9-11].

The most common methods for MF extraction are traditional methods such as Rose-Gotlieb or other liquid-liquid extraction methods. Recently, pressurized (PSE, PLE) or accelerated (ASE) solvent (liquid) extraction methods are commonly used in analyzing TGs and FAs from MF. PSE and ASE methods have several advantages over the traditional methods, like lower consumption of organic solvents, higher efficiency due to high pressure and temperature during the extraction process, automation of extraction, etc.

The most commonly applied methods for detection of animal origin and food fraud related to cheese composition are time consuming or required expensive and specialized instrumentation. This study had the main goal of developing a fast and simple HPLC-based method for TG analysis, retaining satisfactory reliability, and employing low cost and common analytical instrumentation.

## 2. Materials and methods

### 2.1. Chemicals and standards

All HPLC-grade solvents were obtained from Merck (Darmstadt, Germany) and Sigma Aldrich (Germany). Palm fat (PF), bleached deodorized (Sania, Abidjan, Ivory Cost), and anhydrous milk fat (Vitusa, New Jersey, USA) were used in method development. Petroleum ether b.p. 40-60 °C for extraction, n-hexane p.a. and acetone p.a. for extract dilution were from Sigma Aldrich (Germany).

### 2.2. Cheese samples

The total of 130 cheese samples (23 goat cheeses, 98 cow cheeses and 9 cheese analogues) were obtained from across the whole region of the Serbian market. All cheeses were stored in cold storage prior to analysis. Fresh cheeses were predominant, and cheeses in various stages of ripening constituted a minor part of the total cheeses.

### 2.3. ASE extraction

Lipids were extracted from cheese by ASE (ASE 200, Dionex, Sunnyvale, CA) with petroleum ether (b.p. 40-60 °C) at 125 °C and nitrogen pressure of 10.3 MPa in two static cycles of 5 min. Cheese (3-5 g) was weighed, mixed with approximately twice the amount of diatomaceous earth and transferred to 33 mL Dionex extraction cell with glass fiber filters 19.8 mm (Dionex). The extracts were collected in 60 mL glass vials. Solvent was removed under a stream of nitrogen (Dionex Solvent evaporator 500) at 50 °C until dryness. The fat extracts were kept in a desiccator.

### 2.4. Chromatography

Analysis of the TG composition of fats and oils was based on a high performance liquid chromatography (HPLC) method with refractive index detection commonly used for determination of TGs [12]. The chromatographic system consisted of an isocratic pump 1515, autosampler 717, column heater with temperature control module, and refractive index detector 2414 (Waters, Milford, USA). The mobile phase composition was acetone:acetonitrile 64:36 v/v with flow rate of 1 ml/min. Separation of TGs was achieved on two serially coupled Luna C18 columns. Column and detector temperatures were set to 40 °C. Fat extracts were diluted with n-hexane or acetone and filtered through filter discs, pore size 0.22 µm. Sample injection volume was 10 µl.

### 2.5. Statistical analysis

Data preparation for statistical evaluation of results was performed in Microsoft Excel from MS Office. Data for principal component analysis (PCA) was verified by Bartlett's test of sphericity and Kaiser-Mayer-Olkin's test of sample adequacy. For PCA and graphical expression of results, JMP 10 Statistical Discovery (SAS Institute, Cary, USA) software was used.

### 3. Results and discussion

According to our hypothesis that sophisticated, exotic or expensive equipment is not necessary for such analysis, an older HPLC system was chosen with a refractive index detector and the most common C18 reverse phase (RP) column. The developed method is designed for reliable determination of cheese's animal origin and possible adulteration by analyzing the quantity of TGs using equivalent carbon number (ECN) groups, not for precise insight in quantifying individual TGs in MF. Considering this, the employed instrumentation was sufficient to achieve this goal.

#### 3.1. Setting of method parameters

In order to develop a fast and reliable method for determination of TGs in milk and vegetable fat, it was necessary to optimize extraction and chromatographic conditions. Because MF is thermally stable [13] and heating has a great influence on both extraction efficacy [14] and retention time [15], higher temperatures were chosen for MF extraction and chromatographic analysis. Thus, according to optimization results, extraction of all cheese samples was performed at 125 °C in two static cycles of 5 minutes each, and 80 % of each extract volume was flushed into an extraction vial between cycles. The total time consumption for ASE extraction is less than 25 minutes per sample.

To achieve satisfactory separation of TGs within 20 minutes' runtime, after optimization of temperature, mobile phase composition, and flow rate, and after selection of the most suitable chromatographic column, the following optimal conditions were established: column and detector temperatures were 40 °C, mobile phase composition was acetone:acetonitrile 64:36 v/v, flow rate 1 ml/min and two serial coupled C18 columns were used for chromatographic separation of TGs.

#### 3.2. Analysis of cow and goat milk fat extracts

Our optimized method was applied to determine the TG composition of MF. In order to verify that the developed extraction procedure was generally applicable, cheeses with various MF contents were examined. Figure 1 clearly renders the characteristic differences in the constitution of cow and goat MF with regard to TGs. Results confirmed that the greatest difference was in TGs with ECN 38-42, due to differences in the short chain FA content of goat and cow MF [4].

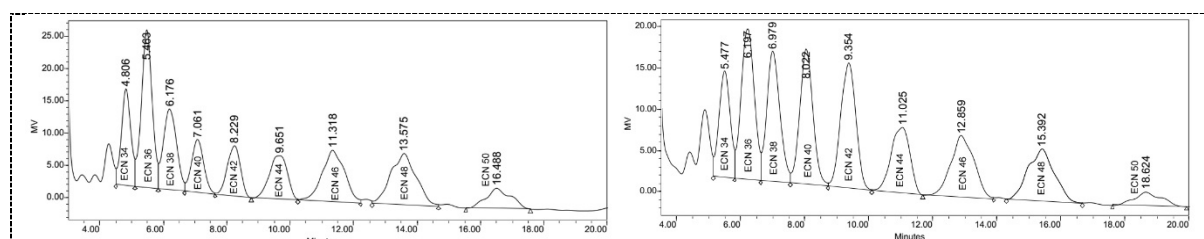
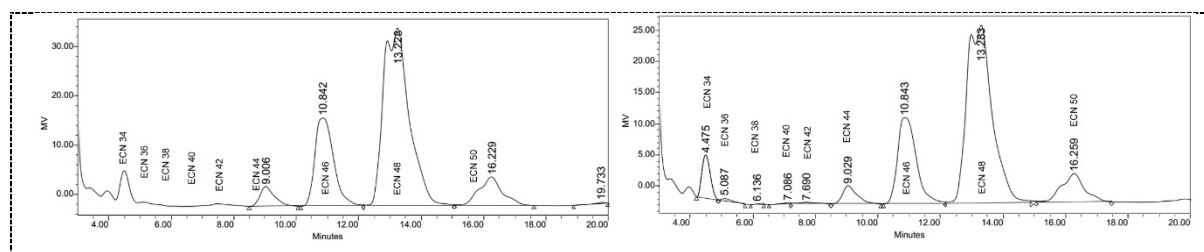
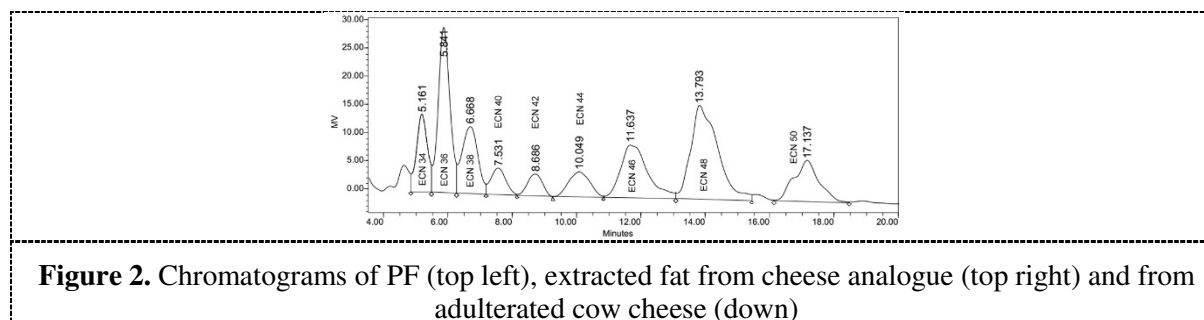


Figure 1. Chromatograms of cow (left) and goat (right) milk fat





**Figure 2.** Chromatograms of PF (top left), extracted fat from cheese analogue (top right) and from adulterated cow cheese (down)

### 3.3. Cheese analogues' TG profile

In the next step, the developed method was tested for its ability to discriminate between natural cheeses and cheese analogues. Results of method as applied to determine the TG profile of cheese analogues showed that PF was utilized to manufacture these products. For illustration and confirmation, commercial PF was analyzed under the same conditions with same method, and its typical chromatogram is shown in Figure 2.

Analysis of cheeses obtained from the Serbian market showed that in a number of our cheese samples, MF was partially replaced with PF. A typical chromatogram of such a cheese is shown in Figure 2. Partial replacement is unequivocally an indicator that economically motivated adulteration (EMA) of cheeses is present on the Serbian market, but until now, it could not be detected or proved. The importance of discovering EMA and its impact on consumers was, in the greatest part, described in the last decade [16].

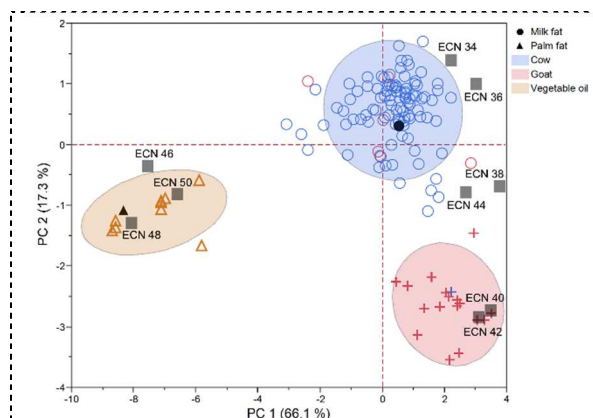
### 3.4. PCA of chromatographic results

PCA on correlations of the chromatographic data produced the first two components that explained over 4/5 total data variance, and corresponding eigenvalues were 5.94 and 1.56. PCA results are shown in Figure 3. Density ellipses cover confidence intervals of 95 %. The Kaiser-Meier-Olsen value was 0.91 and Bartlett's test of sphericity had high significance, with a probability value of  $p < 0.0001$ .

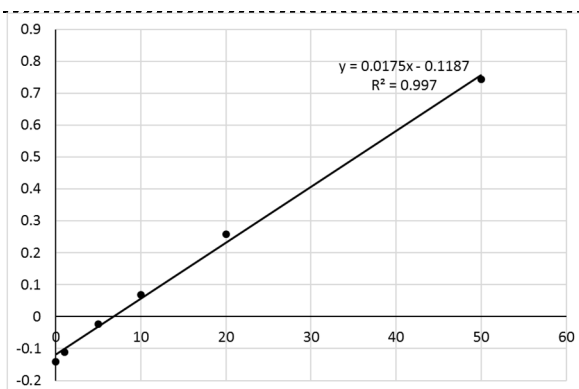
Cheeses were grouped into three clearly separated clusters, based on the TG content of their fat extract as determined by developed method (Figure 3). Chromatographic peak areas of each group of TGs with the same ECNs were integrated. Integrated area data were used as variables in PCA. Data for PF and anhydrous cow MF standard substances are shown as a black triangle and black circle, respectively, in Figure 3. As can be seen, these data could be regarded as central points for corresponding groups of data.

The content of TGs with ECNs from 46 to 50 in the fat extracted from the cheeses was responsible for separating and grouping the cheese analogues, because they predominantly contained PF. TGs with lower ECNs were characteristic for PCA grouping of natural cheeses, in this case TGs with ECNs 34 and 36, and ECNs 40 and 42 for cow and goat cheeses, respectively. TGs with ECNs 38 and 44 were neutral for these groups but of great significance for separating the natural cheeses from the cheese analogues.

PCA results revealed the existence of mislabeled cheeses with respect to animal origin; of these, six cow cheeses were labeled as goat, and one goat cheese was labeled as made from cow milk.



**Figure 3.** PCA of cheese samples from the Serbian market. Blue circles represent cow cheese; red crosses represent goat cheese; orange triangles represent vegetable analogues; black filled circle represents milk fat; black triangle represents palm fat. Mislabelled cheeses are designated by blue crosses or red circles. Grey squares correspond to loading values.



**Figure 4.** Calibration graph for determination of substituted milk fat based on TG analysis.

### 3.5. Method calibration and determination of added fat

A set of MF and PF mixtures with six concentration levels were used for calibration – pure MF, 1 %, 5 %, 10 %, 20 % and 50 % of PF in MF. As shown by PCA and in Figure 2, PF has a significant amount of TGs with ECN from 46 to 50, while MF is richer in TGs with lower ECN. On analyzing chromatograms in our calibration set, the greatest variability in peak area values was obtained for ECN 36 and 48. Quantification of added PF was performed by plotting logarithmic value of ECN 48 to ECN 36 ratio vs. percent of added PF to MF. The linear regression equation is:

$$PF (\%) = 0.0175 \log_{10} \left( \frac{ECN\ 48}{ECN\ 36} \right) - 0.1187 \quad (1)$$

Ratios of TGs with ECN 48 and 36 peak areas, corresponding logarithmic values and recovery data are shown in Table 1.

**Table 1.** Calibration data for estimating the amount of palm fat in milk fat

Palm fat (%)	ECN48/ECN36	$\log_{10}(\text{ECN48/ECN36})$	Palm Fat calculated (%)	Palm fat recovered (%)
0	0.72	-0.14	-1.29	-
1	0.78	-0.11	0.46	46.50
5	0.95	-0.02	5.42	108.48
10	1.17	0.07	10.67	106.73
20	1.81	0.26	21.50	107.49
50	5.53	0.74	49.23	98.47

A very low recovery value was obtained for 1 % PF in MF, i.e., only 46.5 %. However, it is very unlikely that addition of such a small amount of PF would actually occur in EMA, and therefore, this concentration is of minor significance to the relevance of this developed method.

Summarized results of the amount of PF estimated as having been added to the cheeses are given in



Table 2. About 28 % (27 of 98) of cow and 35 % (8 of 23) of goat cheeses included in this investigation were positive for the presence of added PF. Almost double the amounts of PF were detected in cow cheeses than the amounts in goat cheeses.

**Table 2.** Palm fat amounts estimated in fat extracted from cheeses and cheese analogues

Samples	Total	Positive for PF <sup>1</sup>	Lowest % of PF detected	Highest % of PF detected
Cow	98	27	10	16
Goat	23	8	5	8
Vegetable fat	9	-	71	135

<sup>1</sup>PF – palm fat

Vegetable fats extracted from nine different cheese analogues were used as a control for evaluation of method precision and overall accuracy. PF was detected in 97 % of these cheese analogue extracts, with low and high limits given in Table 2.

#### 4. Conclusion

Cheese adulteration, as well as mislabeling of cheese's composition and origin, is present on the Serbian market. Considering the price difference in favor of goat cheese for example, attempts to increase profits by mislabeling are, unfortunately, becoming increasingly common.

The TG composition of goat and cow MF differs sufficiently so that the animal origin of the cheese from which it is extracted can be undoubtedly confirmed based on the results of this developed method of chromatographic analysis. Also, the composition of vegetable fat used for manufacturing cheese analogues has a completely different TG profile, and this can be distinguished from the TG profiles of natural cheeses, both goat and cow. For these reasons, and particularly in conjunction with PCA, this novel, optimized HPLC RID method can be successfully applied to discriminate the goat vs. cow animal origins of cheeses and cheese analogues. The added quantity of vegetable fat can be estimated using the ratio of the content of TGs with ECN 48 and 36 in the extracted cheese fat.

The importance of such rapid and simple food authenticity method development is in providing clear evidence of food composition and origin. This information is of invaluable significance for detecting food fraud and adulteration because, in recent years, such phenomena have become more frequent and have great impact on the public health and economy.

The rapidness of analysis, employment of relatively inexpensive and common equipment and the reliability of the results, compared with other methods used for same purposes, make the developed method suitable for analytical laboratories with limited resources that are required to process large numbers of cheese samples in a short time.

#### Acknowledgment

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## The occurrence of ochratoxin A in kidneys of healthy pigs from Vojvodina province, Serbia

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**Abstract.** Ochratoxin A is a potential contaminant of feed and consequently meat and meat products. Residues of this mycotoxin in meat can pose a food safety issue. Swine production in Serbia and the northern province of Vojvodina is highly developed, since household consumers in Serbia frequently purchase pork. The occurrence of OTA in pig's kidney taken from the slaughter line is a good indicator of the presence of this mycotoxin in meat and meat products. A total of 95 pig's kidneys from Vojvodina, Serbia, were analyzed for ochratoxin A. The results from 19 farms (5 samples per farm from Bačka, Banat and Srem districts of Vojvodina) showed the presence of OTA in 14.74% (14/95 kidneys). The average OTA content was 1.36 µg/kg, median 0.99 µg/kg, and range from 0.10 to 3.97 µg/kg. Results from our research do not suggest any serious problems with OTA contamination in pig's kidneys, but continuous monitoring is needed to avoid any possible future problems.

### 1. Introduction

Ochratoxin A (OTA)-producing fungi are from the genus *Penicillium* and *Aspergillus*. In tropical regions, the most prominent OTA-producing mold is *Aspergillus ochraceus*, while in temperate climate regions, *Penicillium verrucosum* is mostly responsible for OTA [1]. In feeds, OTA is mainly produced by the storage fungi [2] so the management on the farm can have a huge effect on the occurrence of OTA and other mycotoxins.

The Balkan Peninsula region is associated with Balkan Endemic Nephropathy, chronic tubulointerstitial disease associated with the occurrence of OTA in food [3]. The population is directly exposed to OTA through cereals, cereal products, nuts, spices, grape juice, coffee, beer and wine [4]. During a 30-year study of the occurrence of OTA and zearalenone in cereals and feed in neighboring Croatia, a few years with high incidences of these two mycotoxins in cereals were observed. Years with a lot of rain and lower air temperatures produced the highest levels of OTA, up to 68,900 µg/kg. During the rest of the 30 year-period, OTA levels were mostly between 0.26 and 220 µg/kg. However, in the last few years, OTA levels were lower due to dry season droughts [5].

Populations can be also exposed to this mycotoxin indirectly through consuming meat or milk containing OTA. Sub-chronic pig exposure to OTA leads to its accumulation in meat and consequently in meat product [6]. Products of animal origin can contribute up to 3% of the overall human intake of OTA. However, in some cases, animal products might contribute up to 10% of overall OTA, depending on the nutritional preferences of the population [7]. These preferences can



play a vital role in OTA intake, especially if people consume traditional animal products made from animal blood.

In Serbia, the largest meat production sector is pork meat production. In 2017, 307,000 tons of pork was produced [8]. In Vojvodina, the northern province of Serbia, pork is mostly produced at the intensive industrial scale. However, there are small household producers who raise pigs for their own consumption and cure the meat in the traditional way.

In Serbia, average annual household consumption of pork (fresh and frozen) was 45.4 kg, while in Vojvodina, average consumption was slightly higher (47.8 kg) [9]. However, total pork consumption does not just include fresh meat, as other meats are surveyed separately; average annual household consumption of cured meat (Serbia 14.9 kg, Vojvodina 14.8 kg) and processed meats (Serbia 38.9 kg, Vojvodina 40.7 kg) was also reported [9]. A large proportion of these other categories (cured meat, processed meat) are made from pork.

The European Commission regulates the maximum limit (ML) in the European Union for OTA content, 0.05 mg/kg, in complementary and complete feedingstuffs for pigs [10], while in Serbia, the ML of OTA in complementary and complete feedingstuffs for pigs is 0.1 mg/kg [11].

Regulatory limits for OTA in meat and entrails differs among countries. Legislation in Romania and Slovakia for ML of OTA in pig kidney is 5 µg/kg [12,13], while in Denmark, the ML is 10 µg/kg [14] (Jørgensen and Petersen, 2002). About 40% of the level of OTA in kidneys can be found in the meat of the same animal [15]. The Italian regulation for OTA in meat allows a ML of 1 µg/kg [16].

It should be mentioned that OTA in meat products can have differing origins. Some of this mycotoxin can originate from the consumed mycotoxin in feed and this results in OTA residues in the meat. The other origins are from the added spices used in meat processing or from mold growth during the curing process, which can also lead to contamination or production with OTA and/or with other mycotoxins.

After ingestion of feed contaminated with the OTA, 65% is absorbed by the pigs. In the blood, OTA binds to albumin and other macromolecules. The serum half-life of OTA after oral administration is 72-120 h [17]. During the process of metabolism, OTA is accumulated in the tubules of the kidney [18], so the amount of OTA in kidneys can be good indicator of overall exposure of animals to this mycotoxin.

Aside from the problems related to human exposure to OTA residues in meat, OTA can have affect the production parameters in live pigs. OTA added to pig feed at the level of 25 µg/kg reduced the final body weight, average daily gain and feed efficiency, while food intake was not affected at the end of the study [1].

In food and feed production, it is a rare situation that only one mycotoxin is found. This is indeed the situation with OTA, as usually several mycotoxins, the effects of which can be synergistic, co-exist in the food/feed [19]. Very intensive production processes in industrial pig production means animals are at the edge of their biological limits, so even the relatively small amounts of mycotoxins in feed can have dramatic effects on producers' productivity and profitability in the difficult market conditions in Serbia.

## **2. Materials and methods**

### *2.1. Sample collection and preparation*

Pig's kidneys (five from each farm) were collected from 19 pig farms in Vojvodina in the period October-December, 2018 at the two slaughterhouses. The 95 pig's kidneys originated from all three districts in Vojvodina: Banat (4 farms, 20 kidneys), Bačka (10 farms, 50 kidneys) and Srem (5 farms, 25 kidneys). Kidneys originated only from healthy pigs, and on inspection, there were no visual signs of changes on the kidneys. After collection, each kidney was put in a plastic bag, labeled and frozen to -20 °C.

### *2.2. Extraction*

Each entire kidney was blended and a portion of 25 g was extracted with 100 ml of acetonitrile:water (84:16, v/v) using Ultra Turrax T18 homogenizer (IKA, Germany) for 3 min at 11,000 rpm. Crude extract was then filtered through quantitative slow filtration filter paper (Filtros Anioia, Spain). Prior to HPLC analysis, filtered crude extract was cleaned up on MycoSep® 229 SPE columns (Romer Labs, USA).

### 2.3. HPLC analysis

OTA was determined on an Agilent Technologies 1260 Infinity LC system, using an ODS Hypersil column (150 x 4.6 mm, 5µm) (Agilent Technologies, USA). Detection was conducted using a FLD detector at excitation wavelength  $\lambda_{ex}=333$  nm and emission  $\lambda_{em}=470$  nm. The mobile phase was acetonitrile:water (50:50, v/v) with 1% of acetic acid at a flow rate of 1 ml/min. Injection volume was 20 µl, run time was set to 8 minutes, and retention time for OTA was 5.4 minutes. The limit of quantification was 0.10 µg/kg.

## 3. Results and Discussion

The OTA incidences and levels in pig's kidneys originating from 19 farms in Vojvodina are shown in Table 1.

**Table 1.** Occurrence of OTA in pig's kidneys from pigs at slaughter from Vojvodina, Serbia

	Number of samples	>LOQ	%	Average µg/kg	Median µg/kg	Range µg/kg
Bačka	50	10	20.0	1.43	1.42	0.10-3.97
Banat	20	1	5.0	3.93	3.93	-
Srem	25	3	12.0	0.31	0.30	0.10-0.54
<i>Vojvodina</i>	95	14	14.7	1.36	0.99	0.10-3.97

In total, 95 kidneys originating from Vojvodina were analyzed for the occurrence of OTA. The highest number of kidneys was taken from Bačka district, which is the region of Vojvodina with the most intensive agriculture and swine production. The average value in all kidneys in which OTA was above the limit of quantification was 1.36 µg/kg, while median value was 0.99 µg/kg. The overall incidence of OTA in pig's kidneys from Vojvodina was 14.74%. The highest content of OTA in one kidney was 3.97 µg/kg, in a pig from Bačka district. In Banat district, out of 20 kidneys taken from 4 different farms, OTA was found in only one kidney at a level of 3.93 µg/kg.

**Table 2.** The occurrence of OTA on farms in Vojvodina by district (Bačka, Banat and Srem)

	No. of farms	Samples per farm	Farms with no OTA	1 positive sample from farm	2 positive samples from farm	3 positive samples from farm	4 positive samples from farm	5 positive samples from farm
Bačka	10	5	5	2	2	0	1	0
Banat	4	5	3	1	0	0	0	0
Srem	5	5	2	3	0	0	0	0
<i>Vojvodina</i>	19	95	10	6	2	0	1	0

Only on one farm, 4 out of 5 samples contained OTA, and that farm was from Bačka district (Table 2). When farms did stock pigs with OTA in their kidneys, most commonly, OTA was found only in one sample (6 farms in Vojvodina). Ten farms stocked pigs with no determinable level of OTA in their kidneys. None of the participating farms had any previous history of problems related to OTA, so this likely partially explains the relatively low occurrence of OTA. However, on one farm, four out of five

analyzed pig's kidneys contained OTA, which can be a sign of a possible problem. Since OTA is mainly produced by feed storage fungi [2], the management of feed storage can have huge effect on the occurrence of OTA. Also, since the feed storage conditions can affect the OTA, the incidence of OTA can vary from the farm to farm as can be seen in Table 2.

The maximum level of OTA in our pig's kidneys, 3.97 µg/kg, was below 5 µg/kg, the ML for OTA in kidneys regulated by some EU countries (Romania, Slovakia) [13]. However, the presence of OTA in kidney means OTA is likely in meat and meat products derived from these pigs as well. It is obvious the incidence of OTA was very low in the analyzed pig's kidneys. The occurrence of OTA in food and feed depends on climatic conditions and food/feed storage conditions. In Croatia, a few periods with high incidences of OTA and high OTA levels in cereals were reported [5]. It is possible that Serbia, with similar climatic conditions and agricultural production, also has periods with high incidences of OTA, which could lead to OTA in pigs during production or in kidneys or pork.

There are a lot available data on the occurrence of OTA in pig's kidneys, organs, and in meat and meat products in Europe. Many studies were conducted on different types of animal organs, in different types of pig production and in different meat products. In the French monitoring program for OTA in pig's kidneys, less than 10% (out of 300) of kidneys were significantly contaminated with this mycotoxin [20]. In 1997, 1% of pig's kidneys contained from 0.4 to 1.4 µg/kg OTA, while in 1998, 7.6% had the OTA in the range from 0.5 to 5.0 µg/kg [20]. However, in another study from France, out of 70 pig's livers (from three different production systems), OTA was detected in 67% (range from <0.10 to 3.65 µg/kg) [13]. It is known that the distribution of OTA follows the pattern kidney>liver>serum [21]. In a three-year study from Poland, out of 430 animal tissue samples (pig's kidneys, poultry liver and fish muscles), 94 samples were contaminated with OTA in the range from 0.2 to 5.0 µg/kg, 4 samples were in the range 5.0 to 10.0 µg/kg and three samples contained above 10 µg/kg OTA [22]. It was not clear how many of the pig's kidneys contained OTA.

In an Italian report, 31 of 54 analyzed kidneys contained OTA in the range ≤0.05 to ≤0.5 ng/g, while 11 samples had <0.5 to <1 ng/g [16]. Italian guidance for this mycotoxin is in form of the content in meat, so researchers paid attention to those products. A total of 172 salami produced in different regions of Italy were analyzed and OTA was detected in 22 salami, while three salami exceeded the Italian ML for OTA in meat (1 µg/kg) [23]. However, the origin of this OTA contamination was not clear because the mycotoxin can result from the meat, spices or processing. In Croatia, much research was conducted regarding OTA levels in cereals [3, 5, 24] or in finished meat products [25, 26]. In Romania, in a survey on slaughter pigs, the incidence of OTA in kidneys was 79%, with the mean level of 0.54 ng/g, median of 0.40 ng/g and maximum content of 3.18 ng/g [12]. In 2006, 8 out of 10 pig sera taken at the slaughter line from farms in Bulgaria that had problems with nephropathies (enlarged and pale kidneys) contained 28.8 µg/l OTA [27]. In 2007, 9 of 10 samples contained OTA with the mean level of 6.3 µg/l [27]. Previously in Serbia, 33% of pig's kidneys contained OTA in the range from 0.17 to 52.5 ng/g [28]. Another study detected OTA in 70% of pig's kidneys, with mean content of 3.97 ng/g (range 1.3 to 22.0 ng/g) [21].

In Serbia, researchers are not currently focused on OTA, since their attention is oriented toward aflatoxin B1 due to its outbreak in 2012 after heat waves and drought. However, cooler weather with a lot of rain was recorded in 2014, i.e., possible favorable conditions for OTA-producing fungi. There is a need for the continuous OTA monitoring, although our results do not suggest any larger problems. Traditional dried pork products made under domestic conditions might be at higher risk of OTA contamination.

### Acknowledgment

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## Determination of natamycin (food additive in cheese production) by liquid chromatography-electrospray tandem mass spectrometry

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**Abstract.** Natamycin is a polyene macrolide antifungal agent produced by aerobic fermentation of *Streptomyces natalensis* that prevents fungal growth on cheese surface. Commission Regulation EU 1129/2011 establishes a Union list of food additives and the use of natamycin (E235) in production of hard, semi-hard and semi-soft cheese, and lays down maximum residue limit (MRL) of 1mg/dm<sup>2</sup> surface. It also stipulates that natamycin is not to be present at a depth of 5mm and deeper. The aim of this study was to present the analytical method for determination of natamycin in cheese by reverse phase liquid chromatography-electrospray tandem mass spectrometry. Method validation was performed according to Commission Decision 2002/657/EC. The method is linear in the concentration ranges of 0-5 mg/dm<sup>2</sup>, with the limit of detection (LoD) of 0.13 mg/dm<sup>2</sup>. The performance of the method was successfully verified by participating in a proficiency study.

### 1. Introduction

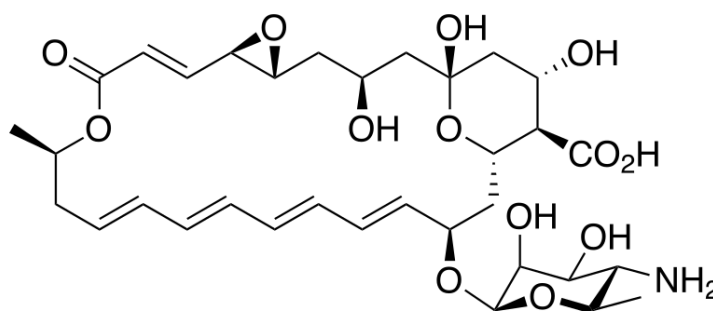
Natamycin (pimaricin), whose formula is shown in Figure 1., is a fungicide of the polyene macrolide group. It shows activity against yeasts and filamentous fungi such as *Candida spp.*, *Aspergillus spp.*, *Cephalosporium spp.*, *Fusarium spp.*, but is not effective against gram-positive and gram-negative bacteria.

In the food industry natamycin is used as an additive (E235) for the preservation of cheese and fermented meat against yeasts and moulds. The mechanism of action of natamycin against moulds is based on inhibition of amino acids and glucose transport through fungal cell membranes due to its specific binding to sterols, principally ergosterol in fungal cell membranes [1]. As it has no effect on bacteria, the starter cultures in fermented food remain active. Natamycin is preferable to other preservatives because it is odorless and colorless and has no adverse effect on the taste of food. The use of natamycin as food additive for surface treatment of hard, semi-hard and semi-soft cheese is regulated by Commission Regulation EU 1129/2011 [2]. The maximum residue level (MRL) is set to 1 mg/dm<sup>2</sup> surface and its presence at a depth of 5 mm and deeper is prohibited. Customs Union Technical Regulation on Safety of Milk and Dairy products - TR TS 033/2013 sets the same MRL [3]. In 2009, EFSA - the European Food Safety Authority, EFSA Panel on Food Additives and Nutrient Sources added to Food (ANS), issued a Scientific opinion on the use of natamycin (E235) as a food



additive [4]. The opinion of the Panel is that proposed use levels of natamycin are not of safety concern if it is only used for the surface treatment of semi-hard and semi-soft cheese and on the casings of certain sausages [4]. The Panel also concluded that there was no concern for the induction of antimicrobial resistance. In 2012., the German Federal Institute for Risk Assessment (BfR) issued a statement that they follow the conclusion of EFSA. Surveys in cheese warehouses and in dry sausage factories where natamycin had been used for up to nine years showed no change in the composition or sensitivity of the contaminating fungal flora [5,6].

However, existence of MRL in relevant legislative, calls upon development and validation of reliable analytical methodology for determination of natamycin in foods for the purposes of regulatory controls.



**Figure 1.** Structural formula of natamycin

Therefore, the aim of this work was the development of sensitive, simple and rapid method for the determination of natamycin in cheese by reverse phase liquid chromatography tandem mass spectrometry.

## 2. Materials and Methods

Natamycin (CAS No. 7681-93-8) analytical standard was purchased from Sigma-Aldrich (St. Louis, USA). Water, methanol, acetonitrile were all HPLC grade and purchased from Sigma-Aldrich. Formic acid LC grade was from Merck (Merck KGaA, Darmstadt, Germany). Stock solution of natamycin,  $c = 1.00 \text{ mg/mL}$  was prepared in methanol and stored at  $4^\circ\text{C}$ .

Natamycin was analysed using Shimadzu UHPLC instrument consisting of LC-30AD pumps, CTO-30A column oven, DGU-20A degasser, SIL-30AC autosampler and CBM-20A system controller coupled to triple quadrupole mass spectrometer LCMS-8040 via an ESI interface (Shimadzu, Europa, Duisburg, Germany). The instrument was controlled by LabSolution software. The analytical column used for separation was Kinetex  $50 \times 2.1 \text{ mm } 2.6\mu\text{m C-18 } 100\text{\AA}$  with UltraGuard cartridge (Phenomenex, Toren, CA, USA). The oven temperature was set to  $40^\circ\text{C}$ . The chromatographic separation was achieved in isocratic mode using 60% of water acidified with 0.1% formic acid (mobile phase A) and 40% of acetonitrile acidified with 0.1% formic acid (mobile phase B) at flow rate of  $0.30 \text{ mL/min}$ . Electrospray ionization (ESI) was used in positive mode, with the following parameters: probe voltage  $4\text{ kV}$ , block heat (BH) temperature  $400^\circ\text{C}$ , desolvation line (DL) temperature  $250^\circ\text{C}$ , interface temperature  $350^\circ\text{C}$ , nebulizing and drying gas flow were 3 and  $15 \text{ L/min}$  respectively. Argon was used as collision gas. The precursor and product ions for natamycin, and collision energies are presented in Table 1.

**Table 1.** Mass spectrometry parameters for natamycin

Compound	Precursor ion (m/z)	Product ions (m/z)	Collision energies (eV)	Ionization mode
		503.2	12	

Natamycin	666.40	485.2	17	ES+
		137.0	41	

Prior to analysis of natamycin in cheese, the cheese sample was divided in two portions, the first containing only the rind (5 mm thick), while the second portion was the layer below the specified depth. From each of the two portions, two samples of 10 g were cut (40x40x5mm). Samples were carefully sliced and cut in small pieces, extracted with 50 mL of 90% methanol in water acidified with 0.01% acetic acid and homogenized with UltraTurrax (T 25 basic, IKA Werke, Germany). The extract (2 mL) was removed and stored at -18 °C for one hour in order to precipitate proteins and lipids. After that, 50 µL of extract was transferred to HPLC vial and diluted up to 1 mL with the initial mobile phase, and 10 µL was injected into LC-MS/MS system. Quantification was carried out using matrix extracted calibrations curves at four levels. With every batch, blank cheese samples were fortified at four different levels with standard solution and subjected to the full extraction procedure.

Validation was performed in accordance to the Commission Decision 2002/657/EC [9]. Each calibration curve was constructed with five concentration levels (including zero) and was fitted to a linear equation within the 0-5 mg/dm<sup>2</sup> range. Linearity was evaluated on three different days. The average regression coefficient ( $R^2$ ) was 0.99957, which was satisfactory. The acceptance criteria were that the average regression coefficient ( $R^2$ ) should be greater than 0.996. Other validation parameters (decision limit CC $\alpha$ , detection capability CC $\beta$ , accuracy, repeatability, reproducibility, measurement uncertainty) were determined based on the procedure given in the software ResVal for the validation of the analytical methods made in EURL RIKILT, Wageningen, The Netherlands. A total of four experiments were performed for four days. The batch of samples was made up of following samples:

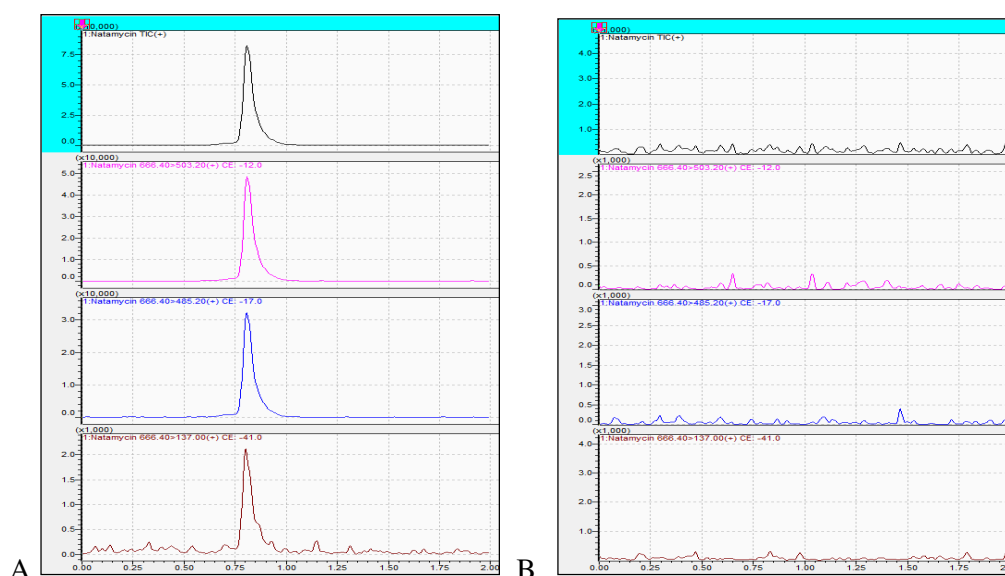
- five calibration level samples (including zero) - fortified blank sample of cheese at 0.0, 0.5, 1.0, 2.0 and 5.0 mg/dm<sup>2</sup>
- seven blank samples
- seven fortified blank samples at half of the required validation level (0.5 mg/dm<sup>2</sup>)
- seven fortified blank samples at the required validation level (1.0 mg/dm<sup>2</sup>)
- seven fortified blank samples at one and a half of the required validation level (1.5 mg/dm<sup>2</sup>)

After validation, method was used in routine laboratory work and forty samples of hard cheese supplied from producers, were analysed using this method.

### 3. Results and discussion

Regarding the analytical methods for the determination of natamycin in food samples, the methods usually involves extraction of natamycin from the sample using organic solvents followed by high-performance liquid chromatography-diode array detection (HPLC-DAD) or liquid chromatography-tandem mass spectrometry (LC-MS/MS) [7,8]. In this work we presented a method with a simple extraction step coupled with efficient separation and highly selective and sensitive detection system. Appropriate extraction with high recovery rate was achieved with 90% methanol in water acidified with 0.01% acetic acid. Addition of the freezing step led to adequate protein and lipid precipitation, so no additional clean-up procedure were required. A suitable chromatographic separation was achieved with a reversed phase (C-18) column and in isocratic elution using 60% of water acidified with 0.1% formic acid and 40% of acetonitrile acidified with 0.1% formic acid at flow rate of 0.30 mL/min. We monitored protonated molecular ion  $[MH]^+$  of 666.4 m/z and three fragments of 503.2, 485.2 and 137 m/z respectively, as presented in Table 1. The most intense fragment of 503.2 m/z was used for quantification. The ratio of abundance of these three fragments was used to conclusively identify natamycin.

Typical chromatograms of blank and cheese sample fortified with natamycin are shown in Figure 2



**Figure 2.** Chromatograms of blank cheese sample fortified with natamycin at level of 1 mg/dm<sup>2</sup> (A) and blank cheese sample (B)

As can be seen there were no interfering peaks at the retention time of natamycin, so matrix endogenous compounds did not affect the method specificity. The limit of detection and quantification were 0.13 mg/dm<sup>2</sup> and 0.26 mg/dm<sup>2</sup> respectively, showing that the developed method had sufficient sensitivity to detect natamycin at the regulatory level (1 mg/dm<sup>2</sup>). The decision limit CC $\alpha$  and the detection capability CC $\beta$  were 1.09 mg/dm<sup>2</sup> and 1.18 mg/dm<sup>2</sup> respectively. The CC $\alpha$  is the limit from which the sample is considered non-compliant with a probability of  $\alpha$  error and CC $\beta$  is the lowest content of the analyte which can be quantified with probability of  $\beta$  error [9]. These limits should be considered in decision making when non-compliant samples are detected. The other validation parameters are shown in Table 2. The results of accuracy, repeatability and within laboratory reproducibility expressed as relative standard deviation (RSD) were satisfactory. Extended measurement uncertainty was 8.5%.

**Table 2.** Validation parameters

Compound	Fortified level (mg/dm <sup>2</sup> )	Accuracy (%)	RSD repeatability (%)	RSD reproducibility (%)
Natamycin	0.5	99.4	3.5	4.7
	1.0	102.4	5.1	5.3
	1.5	101.0	1.9	1.9

The performance of the method was verified by participating in proficiency testing organized by DRRR – Deutsches Referenzbüro für Ringversuche und Referenzmaterialien RVEP 180534 in 2018. A total of 21 laboratories participated in this study, and two samples of cheese were analysed. Z-score values obtained by our laboratory were 0.23 and 0.58 respectively. No false positive nor false negative results were obtained.

This method was applied in everyday laboratory work for the analysis of natamycin in cheese samples. Forty samples of hard cheese, supplied from the producers, were analysed and results are displayed in Table 3.

**Table 3.** Concentration of natamycin (mg/dm<sup>2</sup>) in hard cheese supplied from the producers

		Rind		Internal (≥5mm depth)
Compliant samples	Hard-cheese (n=36)	min.	< LoD	< LoD
		max.	0.670	< LoD
		average	0.455	< LoD
Non-compliant samples	Hard-cheese (n=4)	No.1	6.3	< LoD
		No.2	2.4	< LoD
		No.3	1.67	< LoD
		No.4	4.22	< LoD

Each sample of cheese was divided into two portions as had been described above and subsequently analysed. Of 40 samples of hard-cheese, in four samples (10%) concentration of natamycin exceeded maximum residue level (MRL) in the rind. However, no detectable quantities of natamycin were found in the internal layer, ≥5mm depth. These samples were declared non-compliant. In 36 samples that were declared compliant, concentrations ranged from non-detectable to 0.670 mg/dm<sup>2</sup>. These results were comparable to those found by Molognoni et.al in Brazil [8]. In the samples where natamycin exceeded MRL in the rind, it could be due to unsuitable application of natamycin onto cheese surface. If hard cheeses are preserved by natamycin-soaked wrapping foil, it has to be removed prior to consumption.

#### 4. Conclusion

The method for determination of natamycin in cheese is simple, rapid and has sufficient sensitivity to detect natamycin at the regulatory level, so the method is suitable for routine laboratory work, regulatory controls and efficient tool for compliance with European food legislative.

#### Acknowledgment

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# Problems in determining the nutrition declaration for unpacked meat products – example of domestic cooked sausage

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**Abstract.** This study aimed to determine the degree of change that unpacked pasteurized (i.e. cooked) sausages undergo during their shelf-life. For that purpose, unpacked domestic cooked sausages were examined for basic nutritional parameters, as are required to be stated on labels by local and EU legislation, at the beginning (day 1) and end of their shelf-life (day 40). Results showed the examined parameters varied significantly (the % variance range was 41.5-129.4%), which vastly exceeds the tolerance of variation limits allowed in the legislation (20-50%). The results obtained show the responsible authority would be unable to adequately control the nutrition declaration of these unpacked domestic cooked sausages. The inability to maintain the nutritional content according to the declaration of these unpacked meat products during their shelf-life is a great challenge for quality control of this type of meat product at retail.

## 1. Introduction

Declared nutrition labels need to reflect the nutrient content of the food, be accurate and appropriate for their intended purpose [1]. In accordance with the law in Serbia, a nutrition declaration, indicating nutritional contents, presents information on energy values and quantities of food components, such as: fat (saturated, monounsaturated and polyunsaturated fatty acids), carbohydrate (sugars, polyols, starch), salt, fibre, protein and any vitamins or minerals [2]. The same law lists the mandatory information the declaration must contain, if the nutrition declaration is mandatorily provided. To facilitate implementation of the new legal obligations prescribed for food business operators, the Ministry of Agriculture, Forestry and Water Management published a guide to the declaration of food in June 2018 [3]. They proposed the prescribed deviations, including the measurement uncertainty for the basic nutrition components (Table 1).

Since December 2016, food on the European Union market has been mandatorily labelled with data on the food's nutritional value, and the labelling is the responsibility of food business operators who place food on the market [4]. At the end of 2012, the European Commission prepared a guide [5] on applying unique criteria for acceptable deviations, as provided for by the new European Parliament regulation on the provision of food information and food labelling to consumers [4]. The guide aims to provide consumers with accurate information on food quality through established permitted tolerances,



and to serve both food producers and competent authorities during official food controls. The actual nutritional values of food can differ in relation to the declared values, and therefore, it is essential to define the average nutritional value of the product. The average nutritional value is the one that best represents the amount of nutrients present in the food and takes into account any factors that lead to deviation from the real value.

Unpackaged food is food that is placed on the market without prior packing or is packaged at the point of sale in the presence of the final consumer [2]. In unpackaged foods, the items in the nutrition declaration can be limited to the energy value only, or energy, fat, saturated fat, sugar and salt [5]. However, food business operators are free to implement the full nutrition declaration if they so desire for these unpackaged foods. Moreover, the nutrition declaration for unpackaged foods can be provided either only at delivery of bulk product to retail, or for each consumer unit. Minor deviations in the number of portions and/or consumer units in a product are signalled using the symbol  $\approx$  or  $\sim$  in front of the number of portions and/or consumer units [3].

**Table 1.** Allowable food nutrition component deviations (including measurement uncertainty), based on [5]

Basic component	Allowed deviations
Protein, carbohydrate, sugar, fibre	$< 10 \text{ g na } 100 \text{ g} \pm 2\text{g}$ $10\text{-}40 \text{ g na } 100 \text{ g} \pm 20$
Fat	$< 4 \text{ g na } 100 \text{ g} \pm 0.8\text{g}$ $\geq 4 \text{ g na } 100 \text{ g} \pm 20$
Fatty acids (saturated, polyunsaturated, unsaturated)	$< 10 \text{ g na } 100 \text{ g} \pm 1.5\text{g}$ $10\text{-}40 \text{ g na } 100 \text{ g} \pm 20$
Salt	$< 1.25 \text{ g na/per } 100 \text{ g} \pm 0.375 \text{ g}$ $\geq 1.25 \text{ g na/per } 100 \text{ g} \pm 20\%$
Sodium	$< 0.5 \text{ g na/per } 100 \text{ g} \pm 0.15 \text{ g}$ $\geq 0.5 \text{ g na/per } 100 \text{ g} \pm 20\%$

The tolerances listed in Table 1 include the uncertainty of measurement associated with a measured nutrition component. No further allowance for uncertainty of measurement is made when deciding whether a measured value is compliant with the declared value [5].

Domestic cooked sausages belong to the group of coarse grated boiled sausages and as products, are marketed under various names. Although there is no legally protected name, cooked sausage is a recognizable product on the local Serbian market.

The Serbian rulebook (law) on the quality of minced meat, semi-finished and meat products states that boiled sausages are produced from meat, fat tissue, connective tissue, intestines, blood products and supplements, in which part of the stuffing can form a meat batter and which, after filling into sheaths or coatings, is treated with heat at pasteurization temperature, with smoke [6]. Salt, brine salts, water, spices, spice extracts, sugars, additives, smoke flavourings and natural flavours can be used in these sausages (Table 2). The pasteurized sausages, termed in this study cooked sausages, are cooled after heat treatment and stored at 0 to 4 ° C. Domestic cooked sausages are perishable meat products, and a controlled cold chain is required during their storage.

Recording and reviewing the complete technological process of domestic sausage production is done by defining the quality of raw materials, technological process of production, pasteurization and cooling treatment. The common composition of domestic cooked sausages is listed in Table 2. The origin of the raw materials is local, produced in Serbia.



**Table 2** Common composition of domestic cooked sausages

Raw materials	%	Additives	%
Pork meat, class 2	50	Soybean flour	2
Beef meat, class 2	15	Spices (pepper, onion, garlic, paprika)	2
Pork fat	15	Salt	1.6
Pork offal (liver, heart)	7	Polyphosphate	0.4
Ice	6	TOTAL	100

The aim of this study was to determine the degree of change in declared nutrition contents of unpacked, domestic cooked sausages during their shelf-life.

## 2. Materials and Methods

### 2.1. Technological production process of sausages

Previously salted meat and fatty tissue (with 2% nitrite salt) were minced through a machine with a grinding plate, 3 mm holes. The mince was placed in a cutter bowl and soybean flour and ice were added. These ingredients were chopped in the cutter for five minutes, until the chopped meat/fat reached 11°C. After that, the mosaic components (class 2 pork meat, pork fat) were minced (5 mm) and added to the cutter bowl along with remaining ingredients. All ingredients were then chopped to properly merge them and produce the sausage stuffing. The stuffing was filled into natural casings (pig small intestine, diameter 32-35 mm) and sausages were placed on stroller sticks. After that, raw sausages were heat-treated for 5 h until they reached a core temperature of 70 °C. Heat treatment was conducted using hot air with intense smoke. Cooled sausages were stored under appropriate cold chain conditions (temperature 0-4°C, relative humidity 50-70%). The storage conditions (temperature and relative humidity) were continuously monitored under the manufacturer's HACCP scheme in place.

### 2.2. Sausage sampling

Sausages with a predetermined shelf-life (40 days) were studied. Each sausage weighed a minimum of 200 g and all were from the same production lot. Sausages were sampled twice for determination of compliance with their nutrition declaration. The sausage components were determined immediately after production (day 1; n=12) and at the end of their shelf-life (day 40; n=12).

The following parameters and contents related to the nutrition declaration were determined: energy value (kJ/100g; kcal/100g); salts (%); protein (%); sugar (%); carbohydrate (%); fat (%); total monounsaturated fatty acids (g/100g sausage); total polyunsaturated fatty acids (g/100 g sausage); total saturated fatty acids (g/100 g sausage) and sodium (%).

Variation was calculated using Percentage difference calculator [7].

## 3. Results and discussion

Table 2 presents the average nutritional value and contents of the unpacked sausages at the beginning and end of their shelf-life, as well as the degree of variation among the measured parameters.

**Table 3.** Average nutritional value of unpacked domestic cooked sausages (n=12) at the beginning (day 1) and end (day 40) of their shelf-life and % variation of each measured parameter

Nutritional value or sausage content	Day 1	Day 40	Variation (%)
Energy value (kJ/100g)	1476.5	2350.0	45.7
Energy value (kcal/100 g)	365.8	568.0	43.3
Salt (%)	1.4	3.0	129.4
Protein (%)	17.7	27.4	42.8
Sugar (%)	0.5	0.8	129.4
Carbohydrate (%)	0.5	0.8	41.5
Total monounsaturated fatty acids (%)	15.7	23.9	51.7
Total polyunsaturated fatty acids (%)	3.9	6.6	49.2
Total saturated fatty acids (%)	12.2	20.2	129.4
Sodium (%)	0.6	1.2	45.8
Fat (%)	31.8	50.6	45.8

The results obtained showed the significant variation that occurred in all the examined parameters (the variations were 41.5-129.4%) during the shelf-life of the sausages. This causes a problem in controlling the declared values provided by the food business operator, since the product parameters change significantly during the shelf-life. The loss of moisture from these unpacked sausages caused drastic changes in the product, such that the sausages were almost a completely different product at the end of their shelf-life; certainly the nutrition declaration would have to be completely different on day 40 than the nutrition declaration on day 1. Each of the examined parameters varied by much more than 20%. This level of variation (20%) is prescribed by legal and professional regulations, but our results show the actual variation occurring in this product would pose a serious problem for the responsible authority, the veterinary inspection services. Since the sausage was stuffed in a natural casing and kept unpacked in palettes, we presume it constantly lost water, which disrupted the parameters according to the nutrition declaration.

All food producers are obligated to ensure a high level of consumer protection, which means that food produced must be safe and properly labelled to ensure consumers receive all relevant information about the foods they buy. From December 2016 in the European Union, foods have to be labelled with nutrition data [8]. Nutrient profiling has been defined as “the science of categorizing foods according to their nutritional composition” [9]. For a number of nutrients and foods (total fat, saturated, unsaturated and *trans* fatty acids, protein, carbohydrates, sugars, dietary fibre, salt, fruit and vegetables), population intake goals established in a number of member states are generally consistent (but not uniform) and aimed at preventing major diet-related public health problems in Europe [10].

#### 4. Conclusion

The results of this study clearly show the enormous level of change that domestic cooked sausages undergo during their shelf-life. The inability of the relevant authority to fairly and adequately monitor unpackaged sausages such as these in terms of their nutrition declarations is an insidious problem. It is recommended that unpacked cooked sausages at retail be sold in the shortest possible time, since they rapidly undergo changes during their shelf-life, thus losing any basis for authentication and quality control. Food business operators producing unpacked cooked sausages for retail cannot rely only on a shelf-life study to fully understand their products, since the degree of change that occurs in such products due to moisture loss is substantial. The results obtained show the profound changes in this type of meat product at the end of the shelf-life compared with the characteristics measured at the start, immediately after production. These changes led to completely different characteristics and nutritional contents in

the sausages at the start and end of their retail sale, and this cannot be explained to consumers reading the current nutrition labelling requirements.

### Acknowledgements

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# The effect of essential oils on the color stability of minced meat

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**Abstract.** Meat color is one of the important characteristics for consumers. There are various ways to reduce color deterioration in meat. Among them is application of antioxidants from different sources: fruits, vegetables, herbs and spices. The aim of this study was to conduct a comparative study of the effects of essential oils (peppermint *Mentha spicata* L., citrus (orange) *Citrus sinensis*, cinnamon *Cinnamomum zeylanicum*, rosemary *Rosemarinus officinalis*) on color stability of minced meat. The essential oils at the level of 1.0% were added to minced meat. Meat was held (1) for 15 min under a ultraviolet lamp, or; (2) packaged under vacuum and held for 5 days at 6°C. Then, packages were opened and meat was held for 15 min under the ultraviolet lamp. Ultraviolet light had a great effect on reducing redness of control minced meat. Addition of essential oil promoted protection of the color indicators from deterioration under ultraviolet. However the use of natural antioxidants did not have a significant effect on the stability of yellowness in the meat. The color stability of meat decreased as a result of storage under vacuum mainly due to reduction, except for yellowness. Cinnamon and rosemary oil showed the greatest effect on color stability compared to other antioxidants.

## 1. Introduction

Meat safety and increasing its shelf life are important research tasks. There are various ways to increase the shelf life of products – protective packaging materials, modern types of processing (pasteurization, high pressure) as well as food additives with antimicrobial and antioxidant effects. The oxidation of heme pigments significantly affects the overall acceptability of the finished product, causing the loss of the attractive color of meat and meat products. Meat color is a very important characteristic for consumers, so color assessment is an integral part of meat quality research.

There are many factors that influence the lipid and protein oxidation in meat products: the degree of grinding, temperature storage, the presence of light, the type of packaging, the duration of storage, the presence of antioxidants, etc. In recent years, much research has been focused on the study of natural antioxidants, especially of plant origin and prepared using different extraction methods [1,2]. Plant food ingredients often have a whole range of functional properties: they can inhibit oxidative changes and the growth of microorganisms, while giving the desired color and flavor. Lipid and myoglobin oxidation can be inhibited by the use of essential oils as antioxidants. There are many different sources of natural antioxidants such as fruits (grapes, apple, plum, pomegranate, dog-rose), vegetables (cabbage, radish, broccoli, curry), herbs and spices (rosemary, tea, cinnamon, thyme, mint, oregano) [3-6]. The main components of essential oils are terpenoids and phenylpropanoids [7]. However, some essential oils can have a pro-oxidant effect when they are used in meat in higher concentrations. Antioxidants show significant differences in their effectiveness depending on the type of food product and the conditions of its processing and storage. In this regard, the purpose of this work was to study the effectiveness of the effect of essential oils with antioxidant properties – peppermint, rosemary, cinnamon and citrus on the color characteristics of minced meat.



## 2. Materials and Methods

Pork, *M. Longissimus dorsi*, was taken from female 2-year-old Large White pigs from Russia. Meat was ground through a meat grinder (Vitek, Russia) with a hole diameter of 2-3 mm. The essential oils of peppermint (*Mentha spicata* L.), citrus (orange) (*Citrus sinensis*), cinnamon (*Cinnamomum zeylanicum*), rosemary (*Rosemarinus officinalis*) at 1.0% of the weight of the meat were added to the minced meat. The prepared minced meat was divided into two parts: One part was held for 15 min under an ultraviolet lamp; the second part was packaged under vacuum and held for 5 days at  $6 \pm 2^\circ\text{C}$ . Then, packages were opened and the minced meat was held for 15 min under the ultraviolet lamp.

Determination of the color characteristics of meat products within the CIELab system was carried out using a spectrophotometer (Spectroton, Russia) while simultaneously measuring the reflection coefficients of the samples at 24 fixed wavelengths in increments of 13 nm in the visible spectral range from 380 to 720 nm. Then, mathematical processing of measurement results was carried out by a microprocessor controller in the measuring unit.

To determine the stability of color during storage, the color stability test criterion (U, %) was used. Stability of lightness, redness and yellowness was calculated by the following equation:

$$UL = \left(1 - \frac{|L_1 - L_2|}{L_1}\right) 100$$

$$Ua = \left(1 - \frac{|a_1 - a_2|}{a_1}\right) 100$$

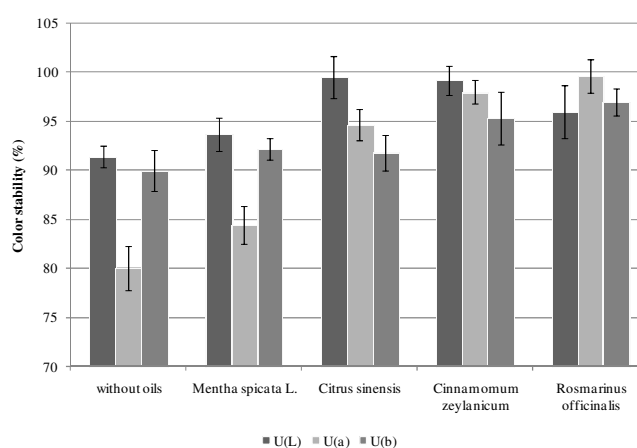
$$Ub = \left(1 - \frac{|b_1 - b_2|}{b_1}\right) 100$$

where:  $L_1, L_2$  – lightness value before and after storage under ultraviolet;  $a_1, a_2$  – redness value before and after storage under ultraviolet;  $b_1, b_2$  – yellowness value before and after storage under ultraviolet

Each experiment was carried out in triplicate. Statistical data processing was performed using Microsoft Excel. The statistical significance of differences between indicators was assessed using Student's t-test.

## 3. Results and Discussion

The results of color stability determinations of minced meat under the ultraviolet light at the start of the study are given in Figure1.

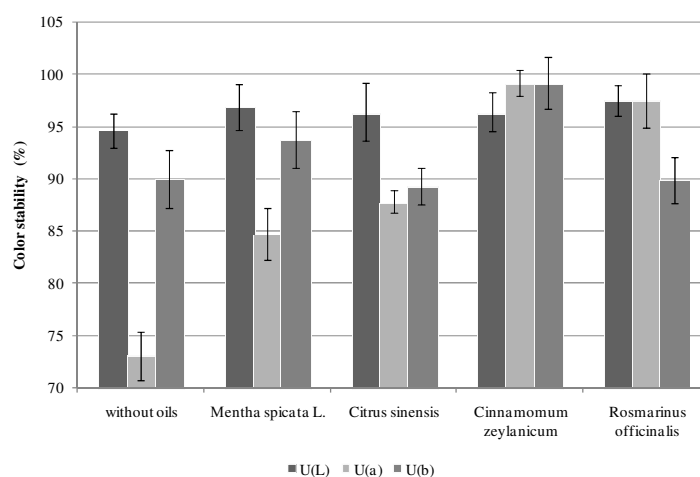


**Figure 1.** The color stability of minced meat on day 1 of the study

According to the results, the effect of ultraviolet on the change in redness was greatest for the control, which is obviously due to the oxidation of myoglobin. The addition of essential oils had a positive effect on the color stability. An increase in the stability of redness by 22.6-24.5% in mince with cinnamon or rosemary oil compared with the control ( $p < 0.05$ ) was recorded. The use of natural antioxidants did not have a significant effect on the stability of yellowness ( $p > 0.05$ ). Essential oil of peppermint was the least effective on color stability ( $p > 0.05$  compared with the control). The aqueous extract of mint leaf produced a decrease in the lightness and redness values and an increase in yellowness value during meat storage [8].

Storage under vacuum for five days had a negative effect on the color stability of all minces except minced meat with added cinnamon or mint (Figure 2). The most stable indicators of redness were recorded for minced meat with added cinnamon or rosemary ( $p < 0.05$ ). In contrast, the yellowness stability underwent no significant changes during storage. Similar data were obtained by [9], who showed that in meatballs, yellowness values were not modified by storage time. According to Fernandez-Lopez *et al.*, rosemary extract increased the stability of the red color compared with the control [9].

The effect of essential oils on meat color can be explained by their minimizing myoglobin and lipid oxidation. Hashemi Gahruie *et al.* indicated that cinnamon and rosemary extracts led to reductions in thiobarbituric acid reactive substances and metmyoglobin in beef burgers [10]. Some analogous results have been published by Yu *et al.*, who indicated rosemary extracts showed significant protection of lipid oxidation and color change in cooked turkey [11]. Similar data were obtained by Valentine *et al.*, who showed that the rosemary-treated ground beef remained redder longer and had lower TBARS values than the untreated control [12]. According Formanek *et al.*, color changes during storage were inhibited by the addition of rosemary extract in ground beef [13]. Sánchez-Escalante *et al.* indicated the powdered rosemary improved the redness of beef patties [14]. In contrast, according Haile, rosemary extract had no significant effect on color stability of liver pate [15]. Yildiz-Turp and Serdaroglu reported no differences during the first days of storage of chicken patties due to the presence of rosemary [16].

**Figure 2.** The color stability of minced meat after 5 days of storage

The effect of adding rosemary essential oil on the oxidative stability of meat depended on the level of added essential oil. Estevez and Cava found that 150ppm rosemary essential oil showed an antioxidant effect, significantly reducing the generation of lipid and protein oxidation products. At higher levels the essential oil had no effect on lipid oxidation while it significantly enhanced the oxidation of proteins and the release of iron from myoglobin [17].

The addition of citrus (orange) oil had a positive effect on stability of redness compared to control ( $p < 0.05$ ), but it was less effective than cinnamon or rosemary oils. According to Fernandez-Lopez *et al.*, rosemary extract proved to be a more effective antioxidant than did orange or lemon extracts in cooked Swedish-style meatballs [9].

#### 4. Conclusions

The use of essential oil increased color stability in minced meat after storage under ultraviolet light. The stability of color indicators decreased as a result of storage under vacuum, mainly due to redness reduction. The yellowness stability did not significantly change during storage. Application of cinnamon or rosemary oils showed greater effects than did citrus (orange) or mint oils. Peppermint oil has the least effect on redness stability of minced meat, among the essential oils studied. Thus, the use of cinnamon or rosemary oil is recommended to protect the color characteristics of minced meat.

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# Mastitis in dairy cow farms in canton Sarajevo and antimicrobial resistance of causative agents

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**Abstract.** Mastitis is frequent and costly disease in dairy farming, while antimicrobial resistance is an important public health threat. Increasing resistance among zoonotic pathogens led to more investigation among animal pathogens. Study, conducted on dairy farms in Canton Sarajevo, aimed to establish mastitis prevalence in dairy cows, causative bacteria and investigate antimicrobial resistance. Lactating animals (n=1214) were tested using the California Mastitis Test during November 2017. Milk from positive animals was microbiologically cultivated. The overall prevalence of mastitis was 9.9 %, while 19 out of 180 dairy farms had at least one mastitis case. In 49.2% of samples, we identified *S. aureus*, 2.5% contained *E. coli*, 0.8% contained *Enterobacteriaceae*, 13.3 % had mixed infection and 34.2% samples had no growth. Using disk diffusion test highest resistances were observed to bacitracin (*E. coli*), trimethoprim/sulfamethoxazole (*Enterobacteriaceae*) and penicillin (*S. aureus*). Since oversight on antimicrobial use in farm animals is sporadic in the country, additional investigations of antimicrobial usage and trends in antimicrobial resistance causing agents are needed. Reducing mastitis rates on farms requires compliance with preventive measures alongside early detection, isolation of cases, culling of repeated cases, microbiological monitoring and testing for antimicrobial resistance before treatment.

## 1. Introduction

Mastitis in dairy farming is the most common and one of the most expensive diseases [1]. Both clinical and subclinical mastitis is found on all dairy farms regardless of the level of cow health management, technology of production, feeding, zoo-hygiene and other factors [2]. Since mastitis control programs primarily focus on contagious agents such as *Streptococcus agalactiae* and *Staphylococcus aureus*, environmental pathogens as causes of mastitis have become more important [3].

On the other hand antimicrobial resistance (AMR) of bacteria is identified globally as the most important public health threat, projected to be a leading cause of human death in the near future [4]. Increased rates and prevalences of antimicrobial resistance among human bacterial pathogens emphasize the need to investigate this phenomenon among animal bacterial pathogens, since many of the microorganisms found in animals are zoonotic.





This paper reports the results of a study conducted on dairy farms in the Canton of Sarajevo (Bosnia and Herzegovina) to establish the prevalence of mastitis, identify causative agents and investigate antimicrobial resistance of the cultured bacterial isolates.

## 2. Materials and methods

The list of dairy farms investigated was compiled using a register of commercial farms maintained by the Veterinary Inspection of the Canton of Sarajevo and expanded to hobby/sustenance farms identified through field surveys by veterinary inspectors. Therefore, we considered 104 registered dairy farms and 76 additional farms with 2707 animals in total. Four farms were excluded from investigation for the reasons of either being closed or the owner refusing to participate. In most of the registered farms, the number of animals was less than was recorded at the time of farm registration (overall 40% fewer animals). Therefore, out of 1472 animals on registered farms, 1011 were lactating cows, whereas of 266 enumerated cattle on additional farms, 203 were lactating cows and so considered for investigation.

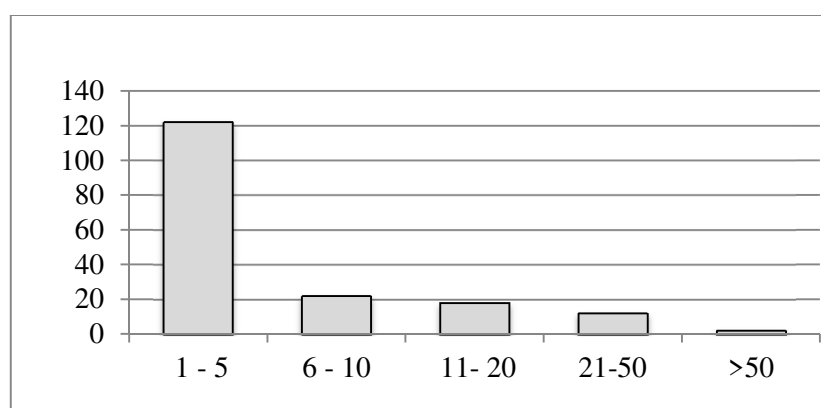
All lactating cows (n=1214) were tested using the California Mastitis Test (CMT) during November 2017. From CMT-positive cows, pooled milk samples from all quarters were taken for microbiological cultivation, before any treatment was administered. Microbiological isolation of bacteria was completed using standardized laboratory protocols [5].

Identified bacterial isolates were then investigated for antimicrobial resistance using the disk diffusion method including following antimicrobials: amoxicillin, amoxicillin/clavulanic acid, ampicillin, bacitracin, cefoxitin, clindamycin, cloxacillin, penicillin G, and trimethoprim/sulfamethoxazole. These results (diameter of inhibition zone) were interpreted using EUCAST recommendations (European Committee on Antimicrobial Susceptibility Testing) as resistant (R), susceptible (S) and intermediate (I).

Data management, analysis and graphical representation of study results were done using Excel (Microsoft Office).

## 3. Results and discussion

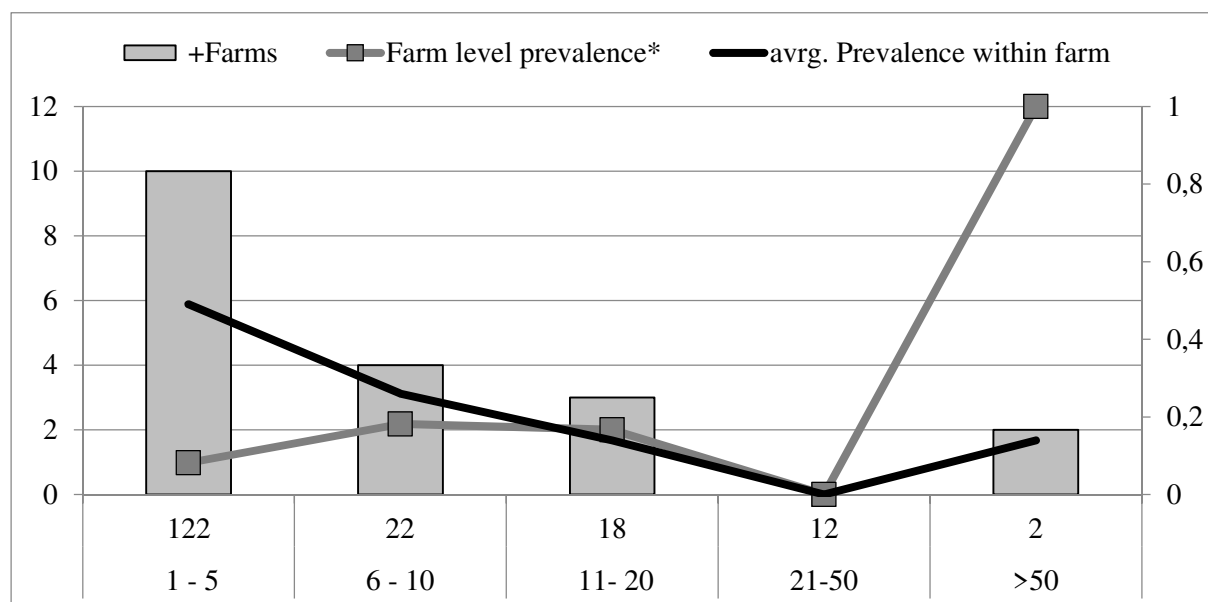
Based on our data, the average farm in the Canton of Sarajevo has 9.3 animals, out of which 6.7 are lactating cows. If the two largest farms are excluded (500 and 99 animals, out of which 430 and 59 were lactating cows, respectively), the average for the remaining farms is 6 animals, or 4 lactating cows per farm (Figure 1).



**Figure 1.** Distribution of investigated dairy farms in Canton Sarajevo (n=176), based on number of animals on the farms

On the basis of CMT results, the overall prevalence of mastitis in dairy cows was 9.8 %, while 19 out of 176 dairy farms had at least one mastitis case (i.e. farm level prevalence was 10.7%). Farm level

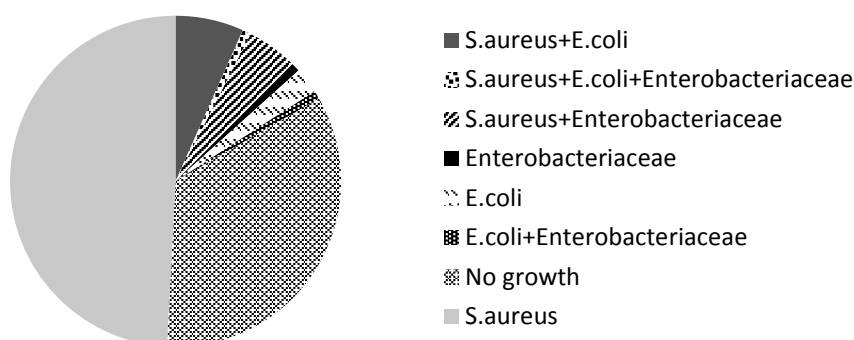
mastitis prevalence was higher among larger farms (>50 animals), while within farm prevalence was higher among small family farms (Figure 2).



**Figure 2.** Number of farms in the Canton of Sarajevo with mastitis cases (blue bars – primary axis), farm level prevalence (orange line – secondary axis) and average prevalence of mastitis within farms (grey line –secondary axis) per farm size (number of animals) group

Even though the established prevalence figures are moderate compared to reported mastitis prevalences in dairy farms from other countries [6-9], there is still room for improvement, especially in small family farms where zoo-hygiene conditions are less adequate.

Microbiological investigation of milks revealed 49.2% contained *S. aureus*, 2.5% contained *E. coli*, 0.8% contained *Enterobacteriaceae* (other than *E. coli*), 13.3% of milks contained mixed infections and in 34.2% of milks, no bacteria were able to be cultivated (Figure 3).



**Figure 3.** Results of the microbiological culturing of CMT-positive dairy cows

A review from 2016 reported the average prevalence of bacterial agents that cause mastitis per 100 cows worldwide [10]. *S. aureus* was responsible for 40-70% of these mastitis cases, while environmental pathogens (i.e. *E. coli*) caused 40% of these [10]. Also, typically 3%–40% of milks from animals with mastitis will not yield any bacterial growth. In animals with chronic or *E. coli* mastitis, the bacterial agent is eliminated by the white blood cells in milk [11].

The disk diffusion test was used to examine AMR in 95 of our bacterial isolates, and high percentages of isolates were resistant to ampicillin (among all three bacterial species/groups), bacitracin (among *E. coli* and other *Enterobacteriaceae* isolates) and penicillin G (among *S. aureus* isolates) (Table 1).

**Table 1.** Antimicrobial resistance in bacteria isolated from dairy cows, given as percentages (%) of resistant (R), intermediate (I) and susceptible(S) bacterial isolates per species/group

Antimicrobial	<i>S. aureus</i>			<i>E. coli</i>			<i>Enterobacteriaceae</i>		
	R	I	S	R	I	S	R	I	S
Amoxicillin	13.6	-	86.4	46.6	-	53.4	42.8	-	57.2
Amoxicillin/clavulanic acid	13.6	-	86.4	26.7	-	73.3	42.8	14.2	43
Ampicillin	39.7	-	60.3	60	-	40	42.8	-	57.2
Bacitracin	17.8	5.4	76.8	86.6	-	13.4			
Cefoxitin	17.8	-	82.2	33.3	-	66.7	28.5	-	71.5
Clindamycin	28.7	24.6	46.7						
Cloxacillin	21.9	4.1	74						
Penicillin G	42.4	-	57.6						
Trimethoprim/ sulfamethoxazole	26	16.4	57.6	40	-	60	57.1	-	42.9

The primary importance of AMR in mastitis-causing bacteria is the resultant human health risk, but simultaneously, reduced efficiency and options for mastitis treatment. Studies report the percentage of penicillin-resistant *S. aureus* is from 17% to 52% (out of the total number of *S. aureus* isolates from milk of mastitis cases) [6,7]. Penicillin-resistant *S. aureus* isolates are found in only 4% of mastitis cases in Norway, where legislation prescribes that only veterinarians decide on and administer antibiotic treatment to animals [12]. In countries where farmers are also legally able to decide on and administer antibiotics, the percentage of resistant bacteria is much higher, up to the point where the antibiotics most commonly used in mastitis treatment are utterly ineffective [12]. Given that Bosnia and Herzegovina is a developing country where oversight of antibiotic use in farm animals is less comprehensive than elsewhere, additional investigations should be conducted to establish trends in AMR in mastitis-causing agents in dairy farms.

Mastitis in dairy cows is a complex disease occurring as a result of the interactions of many factors related to host, causative agent and the environment. Investigations aimed to recognize and specifically target contributing factors has led to the establishment and widespread use of simple mastitis prevention measures such as teat disinfection after milking and dry cow treatments [10]. In order to reduce the occurrence of mastitis in our farms, these standard preventive measures should be fully implemented alongside ensuring early detection of mastitis, isolating diseased animals, culling of repeat cases, microbiological monitoring and testing for antimicrobial resistance before treatment is administered.

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## Changes in chemical attributes during ripening of traditional fermented sausage, “Pirot ironed”

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**Abstract.** “Pirot ironed” is a traditional Serbian dry-fermented sausage manufactured in the south-east of Serbia. The changes in the chemical attributes of Pirot ironed sausage were followed during ripening. Samples were taken on the processing days 0, 7, 14, 21 and 28. Pirot ironed sausage was produced from the most valuable cuts of beef and chevon, without addition of starter cultures or fat tissues. Sausages were manufactured in a traditional drying/ripening chamber, where they were pressed every two days to acquire the typical flat form and to speed up the drying. The final water activity was 0.839. The lowest pH value recorded was 5.30 on the processing day 28. During ripening, the water content decreased significantly from 74.72% to 40.32%, while the protein and the fat amounts increased significantly from 19.12% to 45.79% and from 1.22% to 6.21%, respectively. Up to now, the properties of Pirot ironed sausage have not been recognized or published in scientific literature in spite of the long tradition and popularity of this meat product in Serbia.

### 1. Introduction

Traditional dry-fermented meat products constitute a diverse group of food products. Originating from distinct geographic regions, they bear characteristic sensorial properties gathered in high-quality meat products [1]. “Pirot ironed” (*flattened*) is a dry-fermented sausage manufactured in the municipality of Pirot (south-eastern Serbia). The sausage is traditionally made by mincing and mixing beef, chevon and mutton. Pirot ironed is an artisan Serbian sausage made only from meat and spices (sodium chloride, garlic, hot ground paprika and black pepper) without any additives or starter cultures. After mixing, the meat batter is stuffed into bovine small intestine in units of approximately 4 cm in diameter and 35 cm in length. The product has a characteristic flavour, which is mostly achieved by adding garlic. In the process



of drying, the sausage is pressed with a glass bottle every two days to acquire its typical flat form and to speed up the process. The microbial stability of the sausage is ensured by drying, low winter temperatures and the salt content. The risk in fermented meats is generally considered low because the bacteria numbers decline constantly under the conditions of ripening [2].

Most dry fermented sausages are products with a relatively high fat content, mostly due to addition of pork and fat tissues. The quality of the sausage depends on several factors, among which the meat to fat ratio plays a critical role. Low fat and high protein content of Pirot ironed sausage is achieved by using beef, chevon and mutton of the most valuable meat cuts and with no addition of fat. The sausage is typically manufactured by traditional means in small processing units, so therefore, their chemical attributes can vary. In order to protect the traditional aspect of the product, it is essential to understand the dynamics of changes in chemical parameters during ripening. The objective of this study was to investigate the effect of ripening time on the chemical attributes of Pirot ironed sausage. Up to now, the properties of this type of sausages have not been recognized or published in scientific literature in spite of the long tradition and popularity of this sausage in Serbia.

## 2. Materials and methods

### 2.1. Dry fermented sausages

The study was carried out on 15 dry sausages manufactured in a small-scale facility in Pirot (south-eastern Serbia). Pirot ironed sausage was produced by mincing and mixing the most valuable meat cuts of beef and chevon (50:50). They were trimmed of visible fat. During mixing, the following ingredients were added: sodium chloride, garlic, hot ground paprika and black pepper. The meat batter was stuffed into 37-40 mm diameter bovine small intestine to make horseshoe-shaped sausages of 450-500 g. The sausages were transferred to a traditional drying/ripening chamber where they were kept for 28 days. During the entirety of the drying/ripening period, the sausages were pressed every two days with a glass bottle to acquire the flat form. Once collected, the samples were vacuum-packed and then transported to the laboratory in a refrigerated box. Within this study frame, samples of the sausages were taken on the processing days: 0, 7, 14, 21 and 28. At each processing stage, three samples were analysed in triplicate.

### 2.2. Chemical analysis (pH, water activity, fat, moisture and protein)

In order to prepare the samples for analysis, the sausages were homogenised using a bowl chopper (Blixer 2, Robot Coupe, France). The pH of samples was measured using a digital pH-meter (CyberScan pH 510, Eutech, Singapore). Water activity ( $a_w$ ) was determined using a Fast-lab (Gbx, Romans sur Isère Cédex, France) water activity meter, previously calibrated with sodium chloride and potassium sulphate. Fat content was determined using the ISO method 1444:1996 [3]. Moisture content (%) was determined by weight loss of the sample maintained in an oven (Lenton WF 200, Hope Valley, England) at 105 °C until constant weight (ISO 1442:1997) [4]. Total protein content was determined using the Kjeldahl method (ISO 937:1978) [5] that made use of an Unit 20 digestion block (Tecator™, Foss Analytical AB, Höganäs, Sweden) and automated distillation & titration device (Kjeltec™ 8400, Foss Analytical AB, Höganäs, Sweden). Triplicate determinations of chemical parameters were performed on each tested sausage.

### 2.3. Statistical analysis

For the analysis of the results of chemical traits, one way analysis of variance (ANOVA) was conducted using SPSS package (SPSS 23.0, Chicago, IL, USA). To distinguish statistical differences between the data, Tukey's post hoc test was performed with statistical significance being set at  $P < 0.05$ . Testing of normal distribution for datasets was evaluated by the Kolmogorov-Smirnov and Shapiro-Wilk tests. Correlations between variables were determined using the Pearson's linear correlation coefficient.

### 3. Results and discussion

During the 28 days of ripening, a decrease of pH, from 5.78 to 5.30, was observed (Table 1). The pH values showed significant differences ( $P < 0.05$ ) during manufacture. A decrease of pH during the manufacture is also reported for most other fermented sausages such as Salchichón, Chorizo, Horse Salami and other [6-13]. The initial pH value of Pirot ironed sausage was similar to those found in Salchichón, Chorizo and Horse Salami [6-8]. The decrease of the pH is probably a consequence of the conversion of sugar into lactic acid due to the action of bacteria [14]. At the end of the drying/ripening, pH values of sausages were higher than those reported for Chorizo de cebolla, Chorizo, Salchichón, Salami [15, 16], but lower than those reported for Fuet, Salame Felino and Sremska [16-18], which indicate that fermentation was limited.

**Table 1.** Changes of the chemical composition during ripening of Pirot ironed sausage (mean±standard deviation; n=3)

	Processing time (days)				
	0	7	14	21	28
Chemical composition					
pH	5.78±0.01 <sup>a</sup>	5.71±0.01 <sup>b</sup>	5.69±0.01 <sup>b</sup>	5.58±0.06 <sup>c</sup>	5.30±0.05 <sup>d</sup>
$a_w$	0.956±0.00 <sup>a</sup>	0.945±0.00 <sup>b</sup>	0.905±0.00 <sup>c</sup>	0.868±0.00 <sup>d</sup>	0.839±0.00 <sup>e</sup>
Moisture	74.72±1.0 <sup>a</sup>	65.59±1.40 <sup>b</sup>	54.19±1.31 <sup>c</sup>	43.18±1.08 <sup>d</sup>	40.32±0.70 <sup>e</sup>
Protein	19.12±0.13 <sup>a</sup>	25.24±1.61 <sup>b</sup>	33.82±0.73 <sup>c</sup>	42.71±0.55 <sup>d</sup>	45.79±0.91 <sup>e</sup>
Fat	1.22±0.7 <sup>a</sup>	1.49±0.14 <sup>a</sup>	3.88±0.37 <sup>b</sup>	3.94±0.33 <sup>b</sup>	6.21±2.36 <sup>c</sup>

<sup>a-e</sup> Values in the same row followed by different letters are significantly different ( $P < 0.05$ ).

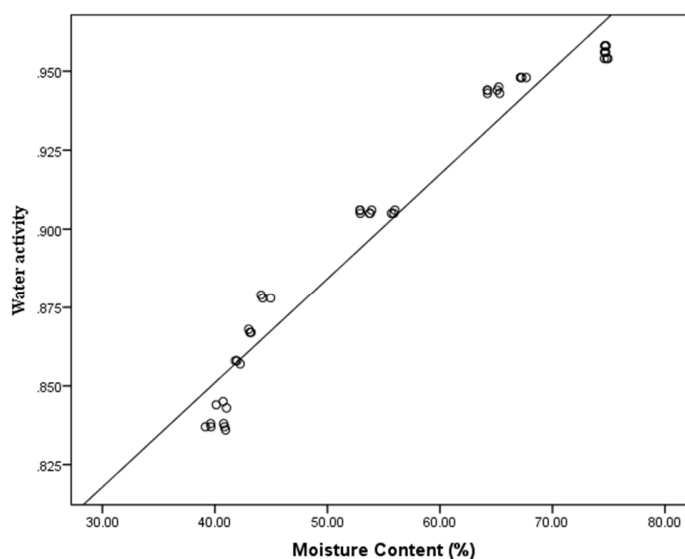
The average moisture content of the sausages decreased progressively during the entirety of the drying/ripening period. The average initial moisture content of the sausages (74.72%) was higher than those found by other authors in Chorizo de cebolla, Salame Felino and Linguiça [15, 17, 19]. This is because Pirot ironed sausage is made with muscle-only meat batters that will, consequently, have more water than other types of fermented sausages made with the addition of fat tissues.

Microbiological indicators of process hygiene in Serbian meat processing establishments are improving after the mandatory adoption of HACCP [20, 21], and likewise, the level of food safety knowledge among Serbian meat handlers is also improving [22]. When using fermentation as a main safety hurdle, most fermented meat products are microbiologically stable when pH 5.3 or lower is obtained within a relatively short period of time. Traditional meat fermentation is anything but fast because it relies on lactic acid bacteria which naturally occur in fresh meat and the environment to initiate the process. While this practice today is perceived as an “art form” in itself, it is highly unreliable as a single meat safety measure. The Pirot ironed sausage has a final pH  $\leq 5.6$ , which coupled with the  $a_w \leq 0.88$  achieved during drying, ensures growth of *Listeria monocytogenes* and *Staphylococcus aureus* is unlikely on this product [23].

Weight loss of sausages is mostly caused by moisture evaporating. Muguerza et al. [24] investigated effects of fat level on processing and quality characteristics of fermented sausages and found that weight loss is significantly affected by the fat level ( $P < 0.001$ ), so the higher the fat level, the lower the weight losses over the same processing time. In consonance with these findings, moisture content of Pirot ironed sausages in our investigation decreased more rapidly during the 28 days of ripening than the values reported for fermented sausages made with addition of pork fat such as Salchichón, Horse Salami, Chorizo de cebolla and Salame Felino [6, 8, 15, 17]. This is because of the low fat content in our samples and the fact that muscles are more capable of losing water, taking it less time to evaporate the same amount of

moisture compared to fat [25]. The other reason for rapid moisture loss is the flattening of the sausages with a glass bottle, making its surface to volume ratio much more favourable for water evaporation. At the end of the ripening process, moisture content values were similar to those found in Salchichón and Salame Felino after 28 days of ripening [6, 17], but higher than those reported for Horse Salami, Chorizo de cebolla and Androlla [8, 15, 26]. As a result of drying, the  $a_w$  gradually decreased to 0.839. Moisture content showed a positive correlation with  $a_w$  values ( $r=0.95$ ,  $P<0.01$ ) (Figure 1), as also reported for Salchichón [6].

Reduction in moisture during ripening caused the increase in fat and protein contents [27], as also observed in our study. The average initial fat content of sausages was lower than those indicated in the literature for Horse Salami, Chorizo de cebolla, Salame Felino, and Androlla [8, 15, 17, 26] and for some traditional Serbian dry-fermented sausages such as Sremska and Sudzuk [18]. At the end of the drying/ripening period, total fat content in the sausage was lower than those reported for Chorizo de cebolla, Salchichón, Fuet, Salami, Salame Felino and Kulen [15-17, 28]. This is a result of addition of well-trimmed beef and chevon and no addition of fat tissues. In consonance with the low fat values, the Pirot ironed sausage showed a very high content of protein, with values higher than those found by other authors for Chorizo de cebolla, Salame Felino, [15, 17] and typical Serbian fermented sausages such as Sremska, Sudzuk and Kulen [18, 28].



**Figure 1.** Correlation between water activity ( $a_w$ ) and moisture content (%) during ripening of Pirot ironed sausage.

#### 4. Conclusion

The microbiological safety of Pirot ironed sausage is ensured by low  $a_w$  values. Use of well-trimmed beef and chevon and no addition of fat tissues results in high protein and moisture contents and, consequently, in low fat content of the sausage. High protein content and flattening of the sausage probably influences the more rapid evaporation of moisture compared to other types of dry-fermented sausages. Consumer awareness of the health benefits of low-fat diets is constantly growing, and therefore, Pirot ironed sausage can be recognized as a low-fat product, which fits their need. Identification and characterisation of autochthonous starter cultures is needed, due to the possibility of industrial production in future.



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## Colour characteristics of vacuum packed fermented sausage during storage

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**Abstract.** The effect of vacuum packaging on colour (instrumental and sensory characteristics) of dry fermented sausage (*Petrovska klobasa*) during storage period was examined. Sausages were dried under controlled conditions and stored unpacked (C) or packed under vacuum (V). The instrumental colour characteristics (CIE  $L^*a^*b^*$  system: lightness –  $L^*$ ; redness –  $a^*$ ; yellowness –  $b^*$ ; hue angle –  $h$  and chroma –  $C^*$ ), sensory evaluation of colour, pH, water activity and moisture content were determined at the end of the drying period (day 0 of storage) and after 30 and 60 days of storage. Vacuum packed sausages had significantly ( $P<0.05$ ) lower  $L^*$  and  $a^*$  on days 30 and 60 of storage compared with  $L^*$  and  $a^*$  values determined at day 0 of storage; however, other instrumental colour characteristics were not statistically different ( $P>0.05$ ). Sausages packed under vacuum had significantly higher ( $P<0.05$ )  $a^*$  value on day 30 of storage and significantly higher ( $P<0.05$ )  $L^*$  value on day 60 of storage compared with unpacked sausages. Also, on both examined storage days, the colour of vacuum packed sausages was more acceptable sensorially than that of unpacked sausages. According to the results obtained in this study, vacuum packaging had a positive impact on colour characteristics (instrumental and sensory) of dry fermented sausages produced in controlled conditions.

### 1. Introduction

Consumers often estimate the quality of meat and meat products based on colour. Colour of meat products is an important parameter in acceptability of products and influences consumers' acceptance of meat products. Oxygen together with illumination cause discoloration of meat products during storage. Therefore, colour formation and colour stability are important quality indicators of fermented dry sausages [1-3].

*Petrovska klobasa* is a dry fermented sausage with designation of origin (PDO) according to Serbian legislation because of its specific and distinctive qualities. This sausage is produced without additives or starter cultures. The intense red colour is one of the important characteristics of *Petrovska klobasa*, and makes it distinctive from other products of the same type. This pleasing colour is formed thanks to the use of red paprika [4].

In order to prevent undesirable oxidation processes and enhance the colour stability of meat products, selected food additives with suitable functional properties, such as nitrates and nitrites, are used in controlled conditions [2, 5]. Since, *Petrovska klobasa* is produced without artificial additives



for enhancing colour characteristics, vacuum packaging could be a suitable packaging method to preserve desirable colour characteristics during longer storage periods [5-7].

Therefore, the aim of this study was to determine effect of vacuum packaging on colour characteristics (instrumental and sensory) of dry fermented sausage (*Petrovská klobása*) during storage.

## 2. Materials and Methods

### 2.1. Preparation of dry fermented sausages

The sausages were produced from minced lean pork meat, pork fat and seasonings (home-made red hot paprika powder, salt, caraway, crushed garlic and sugar). The sausage mixture was stuffed into collagen casings (55 mm diameter). After a rest day, sausages were smoked in by a registered meat processor in Lačarak (near Sremska Mitrovica, Serbia). The smoking process lasted 24 h in controlled conditions. Smoke was produced by a smoke generator using beech wood. Smoking and subsequent drying and ripening processes were performed in an industrial chamber. The average temperature in the industrial ripening room was 7°C, while average relative humidity ranged from 90% to 65%. After smoking, drying and ripening processes were continued until the moisture content was below 35.0%, which occurred day 60 of production. After that, sausages were divided in two groups. Control sausages were unpackaged (C), while other sausages were packed in vacuum (V). All sausages were stored under controlled conditions until day 120 of production (day 60 of storage).

### 2.2. Samples

Samples for analyses were taken on day 0 of storage, i.e. at the end of the drying period (C); day 30 (C1 unpacked and V1 vacuum packed sausages), and; day 60 (C2 unpacked and V2 vacuum packed sausages).

### 2.3. Instrumental determination of colour characteristic

Instrumental colour measurements were performed on fresh cut sausages samples at least 3 cm thickness, using the colorimeter Chroma Meter (CR-400), with aperture of 8 mm in the measuring head and standard additions to measure CR-A33b (Konica Minolta, Japan). Lighting D-65 and standard observer angle of 2° were used. Before each set of measurements, the instrument was calibrated using a white ceramic tile (CR-A43). Sausage colour characteristics were expressed by the CIE  $L^*a^*b^*$  system (lightness –  $L^*$ , redness and greenness –  $a^*$ ; yellowness and blueness –  $b^*$ ) [8]. Hue angle ( $h$ ) and chroma ( $C^*$ ) were calculated using CIE  $L^*a^*b^*$  values [9]. Data presented are means of 10 measurements.

$$h = \tan^{-1} \left( \frac{b^*}{a^*} \right)$$

$$C^* = \sqrt{a^{*2} + b^{*2}}$$

### 2.4. pH, water activity ( $a_w$ ) and moisture content determination

pH and moisture content in *Petrovská klobása* were determined according to respective methods recommended by the International Organization for Standardization [10, 11]. Water activity ( $a_w$ ) in *Petrovská klobása* samples was determined using a Testo 650 instrument with a pressure-tight precision humidity probe (Testo AG, USA). All determinations were made in triplicate.

### 2.5. Sensory analysis of colour

Colour characteristics were also evaluated sensorially on a fresh cut of the sausage samples. Sensory analysis was conducted by a group of six experienced evaluators of different ages, according to a point system for analytical descriptive tests, using a scale from 1 to 5 (5 – optimal colour characteristic; 1 – atypical colour characteristic).

### 2.6. Statistical analysis

Results are presented as mean value±standard deviation. One way ANOVA procedure in Statistica (version 12, StatSoft, Tulsa, USA) was used to compare means. Means were also compared by Duncan's test at the 5% level of significance.

## 3. Results and Discussion

The effect of storage period on the pH,  $a_w$  and moisture content of V and C sausages is shown in Table 1. At the beginning of the storage period, pH,  $a_w$  and moisture content were 5.38, 0.907 and 34.30%, respectively. During the 60 day storage period, pH,  $a_w$  and moisture content significantly decreased ( $P < 0.05$ ) in both groups of sausages, but V sausages had significantly ( $P < 0.05$ ) higher values of these parameters compared to C sausages. Perea-Sanz et al. [5] reported the same trends for  $a_w$  and pH during storage of vacuum packed dry fermented sausages. They concluded the slight but continuous pH decrease which occurred during the storage period could be due to the metabolic activity of lactic acid bacteria, which are likely still active although to a lesser extent. Piras et al. [12] connected lower pH with lipid oxidation in meat products and they also showed that vacuum packaging prevents oxidative processes in dry cured sliced ham. Our results are in agreement with these studies, because during storage, our V sausages had minor changes of the examined parameters (pH,  $a_w$  and moisture content) compared to our C sausages.

**Table 1.** pH, water activity ( $a_w$ ) and moisture content (means±standard deviations) in dry fermented sausage (*Petrovská klobása*) during storage

Parameters	0 day	30 days of storage		60 days of storage	
	C	C1	V1	C2	V2
pH	5.38 <sup>d</sup> ±0.02	5.29 <sup>c</sup> ±0.02	5.25 <sup>b</sup> ±0.01	5.16 <sup>a</sup> ±0.03	5.28 <sup>c</sup> ±0.01
Water activity ( $a_w$ )	0.907 <sup>d</sup> ±0.003	0.873 <sup>b</sup> ±0.002	0.910 <sup>d</sup> ±0.002	0.830 <sup>a</sup> ±0.001	0.877 <sup>c</sup> ±0.001
Moisture content (%)	34.30 <sup>e</sup> ±0.25	23.03 <sup>b</sup> ±0.62	30.28 <sup>d</sup> ±0.16	20.13 <sup>a</sup> ±0.83	28.45 <sup>c</sup> ±0.33

In the same row different letters signify values are significantly different ( $P < 0.05$ )

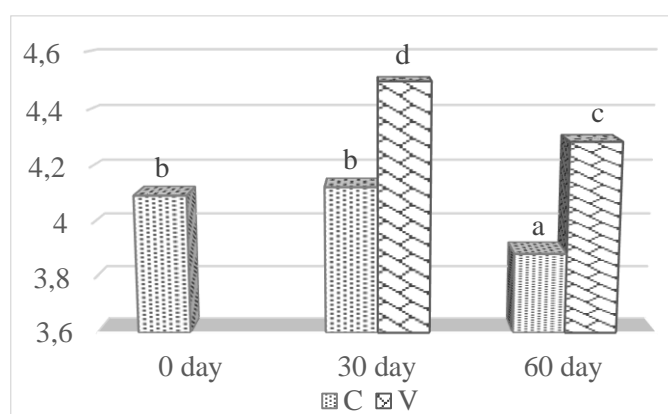
In this study, colour characteristics of dry fermented sausages (*Petrovská klobása*) produced in controlled conditions were determined during 60 days storage. Instrumental colour characteristics were measured on fresh cuts of V and C sausages (days 0, 30 and 60 of storage) (Table 1). During the storage period,  $L^*$ ,  $a^*$ ,  $b^*$ ,  $h$  and  $C^*$  values significantly ( $P < 0.05$ ) decreased in C sausages. V sausages had significantly ( $P < 0.05$ ) lower  $L^*$  and  $a^*$  on days 30 and 60 of storage compared with these values on day 0 of storage. However, other instrumental colour characteristics were not statistically different ( $P > 0.05$ ). Böhner et al. [3] reported that change of  $b^*$  value during storage can be related to the intensity of the oxidation process, and higher oxidation changes lead to increased yellowness, while decreased  $a^*$  could be explained by the reaction of light-induced oxidation of the colour pigments and formation of metmyoglobin. This pigment is grey-brown and likely caused the decreased  $a^*$  on the cut surfaces of our sausages. V sausages had significantly higher ( $P < 0.05$ )  $a^*$  value on day 30 of storage and significantly higher ( $P < 0.05$ )  $L^*$  value on day 60 of storage than did C sausages. Also, at both examined storage periods, V sausages were more sensorially (colourwise) acceptable than were C sausages (day 30 – C1=4.13 and V1=4.50; day 60 C2=3.89 and V2=4.29) (Figure 1). According to these results, vacuum packaging had a positive impact on colour characteristics (instrumental and

sensory) of dry fermented sausages produced in controlled conditions. Similar results were found by other authors. They confirmed the positive impact of vacuum packaging on colour characteristics of meat products during storage [5, 12].

**Table 2.** Instrumental colour characteristics (CIE  $L^*a^*b^*$  system) of dry fermented sausage (*Petrovská klobása*) during storage period; means $\pm$ standard deviations

Colour characteristic	0 day	30 days of storage		60 days of storage	
	C	C1	V1	C2	V2
Lightness ( $L^*$ )	39.32 <sup>c</sup> $\pm$ 2.20	34.39 <sup>b</sup> $\pm$ 2.75	36.54 <sup>b</sup> $\pm$ 1.35	32.02 <sup>a</sup> $\pm$ 2.92	36.48 <sup>b</sup> $\pm$ 2.88
Redness ( $a^*$ )	25.23 <sup>b</sup> $\pm$ 2.34	21.87 <sup>a</sup> $\pm$ 2.28	25.25 <sup>b</sup> $\pm$ 1.82	22.19 <sup>a</sup> $\pm$ 3.81	23.87 <sup>ab</sup> $\pm$ 1.40
Yellowness ( $b^*$ )	25.82 <sup>b</sup> $\pm$ 3.32	22.09 <sup>a</sup> $\pm$ 4.99	20.54 <sup>a</sup> $\pm$ 2.02	21.11 <sup>a</sup> $\pm$ 3.83	21.96 <sup>a</sup> $\pm$ 3.25
Hue angle (h)	45.55 <sup>a</sup> $\pm$ 2.22	44.74 <sup>a</sup> $\pm$ 4.74	41.08 <sup>a</sup> $\pm$ 2.23	43.49 <sup>a</sup> $\pm$ 4.32	42.38 <sup>a</sup> $\pm$ 3.59
Chroma ( $C^*$ )	36.12 <sup>b</sup> $\pm$ 3.81	31.17 <sup>a</sup> $\pm$ 4.90	32.57 <sup>ab</sup> $\pm$ 2.41	30.70 <sup>a</sup> $\pm$ 4.91	32.49 <sup>ab</sup> $\pm$ 2.89

In the same row different letters signify values are significantly different ( $P < 0.05$ )



Values with different letters are significantly different ( $P < 0.05$ )

**Figure 1.** Sensory analysis of colour characteristics

#### 4. Conclusion

During the storage period,  $L^*$ ,  $a^*$ ,  $b^*$ , h and  $C^*$  values significantly ( $P < 0.05$ ) decreased in unpacked sausages. Vacuum packed sausages had significantly lower  $L^*$  and  $a^*$  on days 30 and 60 of storage compared with  $L^*$  and  $a^*$  values determined on day 0 of storage. However, other instrumental colour characteristics were not statistically different ( $P > 0.05$ ). Also, at both examined storage periods, vacuum packed sausages were more sensorially acceptable (colourwise, to a trained panel of evaluators) than were unpacked sausages. In conclusion, vacuum packaging had positive impacts on colour characteristics (instrumental and sensory) of dry fermented sausages produced in controlled conditions.

#### Acknowledgement

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# Myofibrillar fragmentation in entire male, immunocastrated or surgically castrated pigs

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**Abstract.** In order to better characterise differences in meat quality traits between the alternatives to surgical castration, myofibrillar fragment length was investigated in *longissimus dorsi* muscle of entire (n=12), immunocastrated (n=12) and surgically castrated (n=12) male pigs. Higher myofibrillar fragment length was observed in meat from entire pigs than in surgically castrated and immunocastrated male pigs after two days of *post mortem* storage (38% and 19%, respectively). There were no differences between the groups after 7 days of *post mortem* storage. Although this change in myofibrillar fragment length indicates a higher proteolytic potential of muscle from entire male pigs than the other pigs studied, it could not be associated with the meat quality traits of meat tenderness or water holding capacity, suggesting the importance of other influential factors over the proteolysis.

## 1. Introduction

Introduction of alternatives to surgical castration like rearing of entire males (EM) or immunocastrates (IC) is becoming a reality in the European pig sector. In spite of several positive aspects such as better feed conversion, lean deposition and cost-effectiveness [1], rearing of alternatives brings certain disadvantages. In addition to a higher risk of boar taint, these include reduced meat quality, either in terms of lower intramuscular fat (IMF) content, increased meat toughness or reduced water holding capacity in EM and IC compared to surgical castrates (SC) [2]. The literature reports on the differences between the sex categories are inconsistent and need to be further substantiated, as does the aetiology behind the associated biochemical processes. Both EM and IC are metabolically very different categories from SC, due to the presence (in EM) or sudden drop (in IC) of androgen potential [3], which likely affects muscle proteolytic properties known to be associated with meat quality traits like tenderness and water holding capacity (WHC) [4,5]. One of the methods used to indicate post-mortem proteolysis in meat is the measurement of myofibrillar fragmentation [6], which has been applied in the present study in an attempt to characterise changes in EM and IC muscle and is associated with these selected meat quality traits.

## 2. Materials and Methods

The material for the study originated from 36 pigs (12 EM, 12 IC and 12 SC), commercial Landrace × Pietrain crossbred pigs. IC pigs were vaccinated at the ages of 12 and 22 weeks. All pigs were slaughtered at the age of 27 weeks, when their average live weight was 128.9±1.5 kg. A day after slaughter, samples of *longissimus dorsi* (LD) muscles were taken from the cooled carcasses and the next day (day 2), meat quality traits (drip loss, thawing loss, cooking loss, shear force (SF) and IMF)



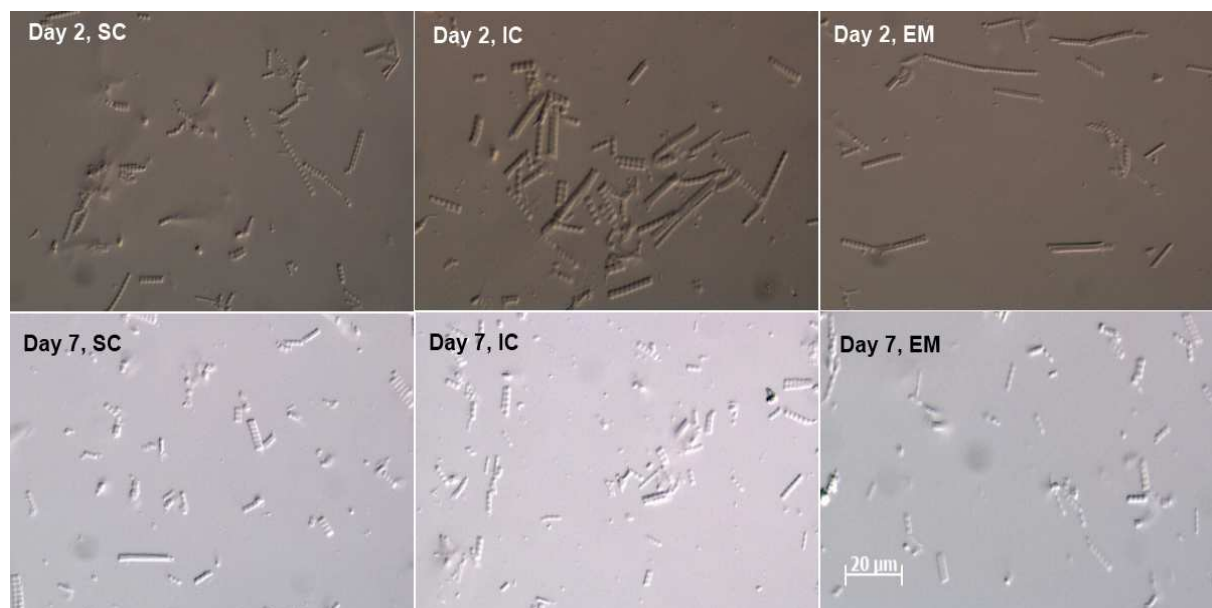
were measured as described in Batorek et al. [7]. On day 2, the length of myofibrillar fragments (MFL) was assessed, and samples of LD were vacuum packed and stored at 4°C for MFL determination 7 days *post mortem* (day 7).

For MFL measurements, a small amount (2.5 g) of LD was excised, cut to small pieces with a scalpel, added to 25 ml of isolation buffer (consisting of 4 mM  $\text{KH}_2\text{PO}_4$ , 16 mM  $\text{K}_2\text{HPO}_4$ , 1 mM EDTA, 1 mM  $\text{NaN}_3$  and 100 mM KCl, at pH 7.0) and homogenised using Ultra-Turrax T25 (IKA Werke GmbH & CO.KG, Staufen, Germany) for 60 seconds at 10.000 rpm. The homogenate was centrifuged at 2°C for 15 min at 1000 g, and the supernatant discharged. Isolation buffer (12.5 ml) was added to the pellet and stirred well; 1 ml of the suspension was further diluted with 12.5 ml of isolation buffer and stirred again. A drop of the suspension was put on a glass slide and examined under a Zeiss Axio Imager Z1 microscope (Carl Zeiss AG, Oberkochen, Germany) with differential interference contrast (DIC) illumination, equipped with Zeiss AxioCam MRc5 digital camera supported by the AxioVision 4.8.2.0 imaging software. For each sample, image analysis (determination of MFL) was performed on 5 different pictures with 40 to 60 fragments measured in each. Relative changes of average MFL (MFL index) between days 2 and 7 were calculated and expressed as %.

Data were analysed with SAS statistical software (SAS Institute Inc., Cary, NC, USA). The effect of sex category was tested using the GLM procedure; when significant differences ( $p < 0.05$ ) were observed, least squared means were compared using Tukey's test. Furthermore, factor analysis (method=prin) was conducted to investigate the relationships between the MFL and the measured meat quality traits.

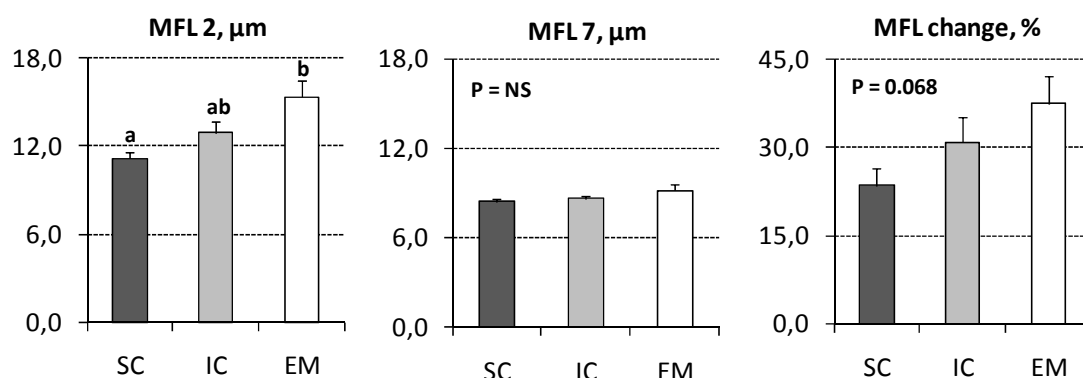
### 3. Results and Discussion

As presented in Figures 1 and 2, MFL in the LD muscles on day 2 was 38% higher ( $p < 0.05$ ) in EM than in SC, while IC had MFL in the intermediate position (19% lower values than in EM), not differing ( $p > 0.05$ ) from either EM or SC. There were no differences ( $p > 0.10$ ) in MFL between the sex groups on day 7. The differences in MFL during the *post mortem* storage (expressed as the MFL index) tended to be higher ( $p < 0.10$ ) in EM (38% MFL reduction) than in SC (24% MFL reduction), with IC being positioned in between (31% MFL reduction).



**Figure 1.** Microscopic images of myofibrillar fragments in *longissimus dorsi* muscle of entire male (EM), immunocastrated (IC) and surgically castrated (SC) pigs after 2 and 7 days of *post mortem* storage.





**Figure 2.** Myofibrillar fragment length on day 2 (MFL 2) and day 7 (MFL 7) *post mortem* and the relative change of myofibrillar fragment length (MFL change) in *longissimus dorsi* muscle of entire male (EM), immunocastrated (IC) and surgically castrated (SC) pigs.

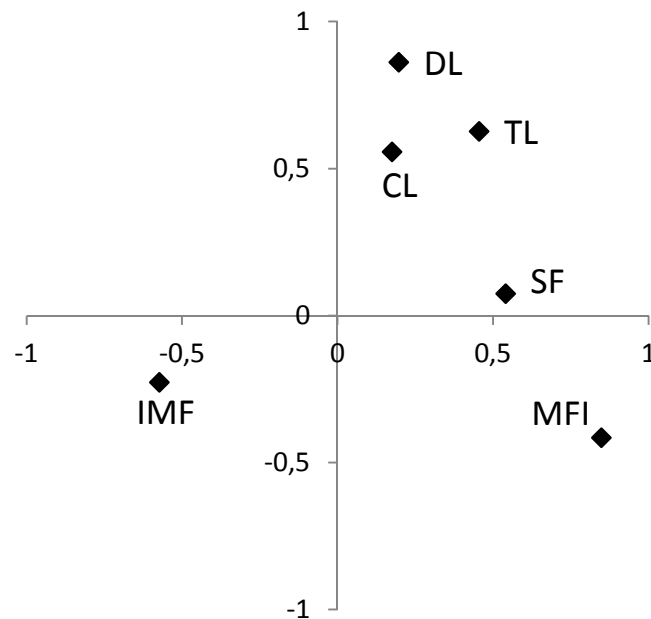
A positive association between the extent of myofibrillar fragmentation and meat tenderness (i.e. lower shear force) was established long ago [8] and is commonly ascribed to *post mortem* proteolytic degradation of the main myofibrillar structural proteins, leading to Z-line disruption [5]. The structural detachments could also release tensions caused by muscular *post mortem* shortening and, thus, improve WHC [4]. The results obtained for the MFL index in the present study indicate the highest proteolytic potential of the LD muscle can be attributed to EM pigs. On the other hand, the difference in MFL on day 2 between EM and SC pig muscle was not accompanied by increases in meat tenderness (SF) or selected WHC traits (Table 1). Moreover, drip loss, thawing loss and shear force did not differ ( $p > 0.10$ ) between our pig castration categories. Only cooking loss was higher ( $p < 0.05$ ) in LD from IC than in SC pigs. Factor analysis (Figure 3) confirmed a lack of association between MFI and our measured meat quality traits, while WHC traits were positively related to each other and negatively related to IMF. Similarly to the present research, our recent study on EM and SC differences in muscle physical-chemical traits and proteomic profile [9] also indicated a higher degree of proteolysis in EM (based on the higher abundance of myofibrillar protein fragments) and no correlation with meat toughness or WHC. This suggested that other traits, like IMF and protein oxidation, could be the main factors in explaining the differences in meat quality of LD muscle derived from EM and SC pigs.

**Table 1.** Meat quality traits measured in *longissimus dorsi* muscle

Trait	EM <sup>a</sup>	IC <sup>b</sup>	SC <sup>c</sup>	RMSE <sup>d</sup>	<i>p</i> -value
Intramuscular fat	1.2 <sup>x</sup>	2.0 <sup>y</sup>	2.2 <sup>y</sup>	0.66	0.001
Drip loss, %	3.8	4.1	3.8	2.51	0.450
Thawing loss, %	10.6	8.7	8.0	3.02	0.123
Cooking loss, %	30.7 <sup>xy</sup>	32.7 <sup>y</sup>	29.3 <sup>x</sup>	2.68	0.015
Shear force, N	63.8	68.3	60.0	10.39	0.166

<sup>a</sup> Entire males; <sup>b</sup> Immunocastrates; <sup>c</sup> Surgical castrates; <sup>d</sup> Root-mean-square error;

<sup>x, y, z</sup> Within a row, values with a different superscript differ significantly ( $p < 0.05$ ).



**Figure 3.** Factor analysis plot showing associations between selected meat quality traits (shear force – SF, intramuscular fat – IMF, drip loss – DL, thawing loss – TL, cooking loss – TL) and myofibrillar length index (MFI)

#### 4. Conclusions

Based on the myofibrillar fragment length, the present study indicates that LD from EM pigs possesses the highest proteolytic potential among the pig castration categories studied. The results on myofibrillar fragment length, however, could not be associated with meat tenderness or water holding capacity, implying that other factors have more impact. Further research on characteristics like protein oxidation and enzyme activities are needed to explain the nature of the underlying processes.

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## The effect of winter savory (*Satureja montana* L.) extract on the quality of cooked pork sausages

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**Abstract.** The effect of winter savory (*Satureja montana* L.) supercritical extract on pH, Thiobarbituric acid reactive substance value, microbiological profile and sensory attribute of flavour was examined. All four tested concentrations of supercritical fluid extract (0.025, 0.050, 0.075 and 0.100 µL/g) resulted in significant ( $p < 0.05$ ) reduction of thiobarbituric acid reactive substances and inhibition of microbial growth. The addition of supercritical fluid extract had no negative impact on flavour. Therefore, winter savory extract could be successfully applied as natural antioxidant and antimicrobial agent in order to improve quality of cooked pork sausages.

### 1. Introduction

Cooked sausages are broadly consumed meat products in Serbia [1]. During different stages of processing, distribution and storage, cooked sausages undergo chemical (lipid and protein oxidation), microbiological and sensory deterioration [2,3]. One of the main strategies to slow down deterioration of cooked sausages is the use of synthetic antioxidants and antimicrobial agents. On the other hand, these compounds are recognized as potentially unhealthy owing to their carcinogenic properties [1-3].

Therefore, the replacement of synthetic antioxidants by natural antioxidants/antimicrobials, including plant essential oils and extracts has been proposed in different type of meat products [1, 2, 4, 5].

Winter savory (*Satureja montana* L.) has been designated as an important aromatic plant broadly spread in the Balkans. Due to the high content of monoterpenes polyphenols, thymol and carvacrol, winter savory and its extracts can be used as aroma additives with functional potential in food and pharmaceutical industry products [6]. Conventional extraction techniques (Soxhlet extraction with organic solvents and hydrodistillation) were predominantly used for obtaining *Satureja montana* L. essential oil and volatile extracts. On the other hand, these methods retain various drawbacks: low extraction efficiency, longer period of extraction, solvent residues in the extracts which decrease their quality and have negative influence on the environment due to huge consumption of organic solvents [7,8]. In order to overcome these disadvantages and achieve high quality and high bioactivity of extracts, novel extraction methods, including supercritical fluid extraction, have been developed [8]. There are no studies in the available literature concerning the application of supercritical fluid extracts of *Satureja montana* L. in meat products. Therefore, the aim of this work was to assess the effect of supercritical fluid extract (SFE) obtained from winter savory (*Satureja montana* L.) on pH, oxidative and microbiological stability, and sensory properties of cooked pork sausages during refrigerated storage.



## 2. Materials and Methods

### 2.1. Plant material

Winter savory (*Satureja montana* L.) was produced at the Institute of Field and Vegetable Crops, Novi Sad, Serbia. The collected plant material (aerial parts) was air dried and stored at room temperature. Plant material was grounded in a domestic blender and the mean particle size of raw material (0.377 mm) was determined using sieve sets. Moisture content of plant material (9.71%) was analysed using the standard procedure, i.e., by drying the plant material at 105 °C until constant weight.

### 2.2. Supercritical fluid extraction

The supercritical fluid extract was obtained using a laboratory-scale high pressure extraction plant (HPEP, NOVA, Swiss, Efferikon, Switzerland) described in Šojić *et al.* [8]. Plant material (50.0 g) was placed in an extractor vessel and extraction process was carried out at 100 bar, 40 °C, for 4 h using 0.2 kg/h CO<sub>2</sub> flow rate. Solvent-free SFE was recovered in a separator under following conditions: 15 bar and 25 °C. Total extraction yield was measured and result was expressed as grams of total extractable compounds per 100 grams of plant material (g/100 g), i.e. percentage (%).

### 2.3. Preparation of cooked pork sausage

Cooked pork sausages were created in a local industrial meat company (Strand, Novi Sad, Serbia). The sausage batter consisted of meat from pork shoulder (50%), pork back fat (15%), pork skin emulsion (15%), ice water (15%), soy protein (2%), nitrite salt (2%) and spice mix (Lay Gewurze OHG, Germany) (1%). Procedure was described in details by Šojić *et al.* [1]. SFE was added to the sausage batters at concentrations of 0.025 µL/g (SFE1), 0.050 µL/g (SFE2), 0.075 µL/g (SFE3) and 0.100 µL/g (SFE4). SFE was mixed with salt and added to sausage batters prior to stuffing. The remaining batch (without SFE) was assigned as the control sausage type (C). All sausages were stuffed into artificial casings (Ø ≈ 36 mm) and pasteurized until an internal temperature of 70°C was reached. Immediately after the heating process, sausages were cooled and stored in a cooling chamber (to 4°C) until analysis.

### 2.4. Sausage sampling

Samples taken at distinct periods of storage were three randomly selected sausages from each sausage group after 0, 15 and 30 days. Analyses were carried out on the day of sampling, and were completed in duplicate for each day of sampling.

### 2.5. pH determination

The pH of sausages was measured using the portable pH meter Testo 205 (Testo AG, USA) equipped with a combined penetration tip with temperature probe. The pH meter was calibrated before the readings using two buffer solutions (pH=4.00±0.05 and pH = 7.00±0.01 at 20±2 °C).

### 2.6. TBARS determination

2-Thiobarbituric acid reactive substances (TBARS) were determined as described by Šojić *et al.* [1]. TBARS were expressed as milligrams of malondialdehyde (MDA) per kilogram of sample.

### 2.7. Microbiological analysis

Microbiological analyses were performed on three samples from each group of the cooked pork sausages in duplicate. Twenty grams of sausage sample were homogenized for 10 minutes at 200 rpm (Unimax 1010, Heidolph, Germany) in 180 mL 1 g/L buffered peptone water (Merk, Darmstadt) and then serial decimal dilutions were prepared (up to 10<sup>-3</sup>). One millilitre of each dilution was placed in a sterile Petri plate and overlaid with appropriate media depending on the type of tested microorganism. The following microbial analyses were performed: total number of aerobic mesophilic bacteria (TBC), *Salmonella* spp., *Escherichia coli*, *Listeria monocytogenes* [8]. Results were expressed as a cfu/g.

### 2.8. Sensory analysis

The Difference-Control-Test was carried out by 10 trained sensory assessors, who were able to discriminate sausages in relation to the investigated attributes (flavour). Panellists were asked to evaluate the control sausage first and then to determine how different the other coded sausages were from the control by rating this difference on a scale from 0 to 6, where 0 = no difference; 1 = very slight difference; 2 = slight/moderate difference; 3 = moderate difference; 4 = moderate/large difference; 5 = large difference; and 6 = very large difference [1].

### 2.9. Statistical analysis

Statistical analysis was carried out using STATISTICA 12.0 (StatSoft, Inc., Tulsa, OK, USA). All data were presented as mean values with their standard deviations (mean±SD). Variance analysis (ANOVA) was performed, with a confidence interval of 95% ( $p < 0.05$ ). Means were compared by Fisher's LSD test.

## 3. Results and Discussion

The effect of SFE on the pH of cooked pork sausages is shown in Table 1. At the beginning of storage, pH ranged from 6.33 to 6.36. Storage period significantly ( $p < 0.05$ ) affected the decrease of pH. Significant drops of pH were registered in each sausage group during 30 days of refrigerated storage. Most probably, this was the result of growth and metabolic activity of lactic acid bacteria [3,9]. Lipid oxidation was estimated by determining the ranks of TBARS (mg malondialdehyde/kg) (Table 1). The initial TBARS values varied from 0.09 mg malondialdehyde/kg (SFE1; SFE3) to 0.12 mg malondialdehyde/kg (control). During the storage period, TBARS values significantly ( $p < 0.05$ ) increased for all treatments. Most probably, this was the result of lipid oxidation [10]. After 15 days of storage, all four concentrations of SFE significantly ( $p < 0.05$ ) affected the reductions of TBARS values.

Additionally, at the end of storage (day 30), the TBARS levels varied between the treatments in the following order: C>SFE1≥SFE2≥SFE3>SE4. These results indicate the strong antioxidative effect of SFE. Antioxidant activity of SFE could be attributed to the presence of its major monoterpene phenolics, particularly carvacrol (67.58%) [6]. A similar result was observed by de Oliveira *et al.* [2].

**Table 1.** pH and 2-thiobarbituric acid reactive substance values of cooked pork sausages

Day of storage	Control	SFE1	SFE2	SFE3	SFE4
<i>pH</i>					
0	6.33±0.02 <sup>Aa</sup>	6.34±0.02 <sup>Aa</sup>	6.35±0.01 <sup>Aa</sup>	6.36±0.02 <sup>Aa</sup>	6.35±0.02 <sup>Aa</sup>
15	6.32±0.04 <sup>Aa</sup>	6.33±0.03 <sup>Aa</sup>	6.33±0.04 <sup>Aa</sup>	6.32±0.03 <sup>Aa</sup>	6.36±0.02 <sup>Aa</sup>
30	6.07±0.05 <sup>Cb</sup>	6.18±0.03 <sup>ABb</sup>	6.20±0.05 <sup>Ab</sup>	6.12±0.04 <sup>BCb</sup>	6.15±0.03 <sup>ABb</sup>
<i>2-Thiobarbituric acid reactive substances (mg malondialdehyde/kg)</i>					
0	0.12±0.02 <sup>Ac</sup>	0.09±0.04 <sup>Ac</sup>	0.10±0.01 <sup>Ac</sup>	0.09±0.03 <sup>Ac</sup>	0.10±0.02 <sup>Ac</sup>
15	0.35±0.01 <sup>Ab</sup>	0.32±0.01 <sup>Bb</sup>	0.29±0.01 <sup>Cb</sup>	0.30±0.02 <sup>BCb</sup>	0.25±0.02 <sup>Db</sup>
30	0.54±0.04 <sup>Aa</sup>	0.43±0.02 <sup>Ba</sup>	0.42±0.03 <sup>Ba</sup>	0.39±0.02 <sup>Ba</sup>	0.39±0.02 <sup>Ba</sup>

Values with different letters (<sup>A-D</sup>) in the same row are significantly different ( $p < 0.05$ ); Values with different letters (<sup>a-c</sup>) in the same column are significantly different ( $p < 0.05$ ); SFE – supercritical fluid extract.

The microbiological profile of cooked pork sausages during 30 days of storage under refrigeration is shown in Table 2. The addition of SFE significantly ( $p < 0.05$ ) reduced the total number of aerobic mesophilic bacteria (TBC). At the end of storage, TBC was significantly ( $p < 0.05$ ) different between the sausage groups, in the order: C>SFE1≥SFE2>SFE3>SFE4. It should be underlined that the antimicrobial potential of SFE can be mainly attributed to the presence of carvacrol, thymol and eugenol [7].

**Table 2.** Total number of aerobic mesophilic bacteria (cfu/g) of cooked pork sausages

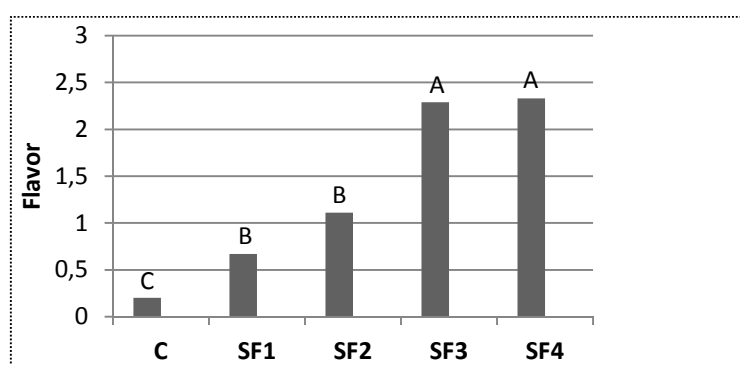
Day of storage	Control	SFE1	SFE2	SFE3	SFE4
0	66.7±15.3 <sup>Ac</sup>	46.7±5.8 <sup>Bc</sup>	43.3±5.8 <sup>Bc</sup>	43.3±5.8 <sup>Bc</sup>	16.7±11.6 <sup>Cc</sup>
15	157±12 <sup>Ab</sup>	116±6 <sup>Bb</sup>	100±10 <sup>BCb</sup>	93.3±5.8 <sup>Cb</sup>	83.3±5.8 <sup>Cb</sup>
30	710±36 <sup>Aa</sup>	520±10 <sup>Ba</sup>	500±20 <sup>Ba</sup>	426±31 <sup>Ca</sup>	323±25 <sup>Da</sup>

Values with different letters <sup>(A-D)</sup> in the same row are significantly different ( $p < 0.05$ ); Values with different letters <sup>(a-c)</sup> in the same column are significantly different ( $p < 0.05$ ); SFE – supercritical fluid extract.

The initial TBC ranged from 16.7 cfu/g (SFE4) to 66.7 cfu/g (C). As expected, for all treatments the TBC significantly ( $p < 0.05$ ) increased during 30 days of storage. Three analysed foodborne pathogenic bacteria (*Salmonella* spp., *E. coli*, *L. monocytogenes*) were not detected both in control and treated sausages.

Sensory panel results for flavour assessment are shown in Figure 1. All four concentrations of SFE significantly ( $p < 0.05$ ) affected sausage flavour. The intensity of flavour was very slight (SFE1; SFE2) and slight/moderate (SFE3; SFE4), i.e. different to control sausages.

Hence, the results obtained in this study indicate the use of SFE (0.025-0.100  $\mu\text{L/g}$ ) had a relatively mild sensory effects on sausage flavour.



Values with different letters (A-C) are significantly different ( $p < 0.05$ )

**Figure 1.** Sensory properties of cooked pork sausages

#### 4. Conclusion

In conclusion, application of winter savory supercritical fluid extract (SFE) retarded lipid oxidation and reduced microbial growth, with only a very slight to slight/moderate alteration of the original flavour of cooked pork sausages. Hence, these results indicate that winter savory supercritical extract (SFE) could be successfully applied as a natural plant antioxidant and antimicrobial agent in cooked pork sausages.

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# Trace elements and heavy metals in multifloral honeys from Serbia

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**Abstract.** This study was to determine the contents and any correlations of As, Cu, Zn, Fe, Cd and Pb in multifloral honeys. Honey, among other bee products, is a good bioindicator since it can reveal the connections between soil, plants and honeybees. Ninety-two samples of multifloral honey were collected from the retail market during the 2018 vegetation season and analyzed to determine mineral content. Analysis of the elements was performed by inductively coupled plasma mass spectrometry (ICP-MS). The most abundant element was Fe, with average concentration of  $2.21 \pm 1.00$  mg/kg, followed by Zn, Cu, Pb, As and Cd. The results obtained show positive correlations: Zn-As, Fe-As, Fe-Cu, Fe-Cd, Cd-Cu and Cd-Pb. Negative correlations are noticeable between Pb and all other minerals except Cd.

## 1. Introduction

Honey is the natural sweet substance produced by honey bees (*Apis mellifera*) from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature [1]. Honey as food is an important source of energy and also has multiple healing properties, whether it is floral honey, or honeydew honey.

About 90-95% of the dry matter of honey is sugar, followed by water, in which organic acids and mineral compounds are dissolved. The numerous constituents of honey include enzymes, amino acids, organic acids, carotenoids, vitamins, minerals, and aromatic substances. Honey is rich in flavonoids and phenolic acids that exhibit a wide range of biological effects and act as natural antioxidants [2]. The composition, color, aroma and flavor of honey depend mainly on the flowers, geographical region, climate and honeybee species involved in its production, and are also affected by weather conditions, processing, manipulation, packaging, and storage time [3]. Honey contains components that have antioxidant activity, and also, honey is useful because of its antimicrobial components [4,5]. Mineral content determination in honey is important because honey is a valuable dietary supplement. Trace elements in honey are valuable as indicators of geographical origin. Some heavy metals are very toxic, so it is important to determine their content from the food safety point of view. There are no defined criteria for most of the minerals in honey. As an important ingredient of many dietary supplements, honey should meet requirements regarding Pb content (6).

Nevertheless, honey is good bioindicator, so it is possible to use analysis of honey to determine the relationships of minerals in soil, plants and bee products. Some of the minerals co-exist in positive relationships with plants, nectar and bee products, respectively. It is possible to determine relationships





between the occurrences of different elements in certain bee products [7]. Multifloral honey, the most common honey type in Serbia, was chosen for this study [8,9]. The aim of this study was to determine the contents of As, Cu, Zn, Fe, Cd and Pb in multifloral honey as well as their correlations.

## 2. Materials and Methods

Ninety-two samples of multifloral honey were collected from the market during one vegetation season (from March until November 2018) and analyzed to determine mineral content, particularly some trace elements and heavy metals commonly found in honey. Honey samples were kept at 4°C until analysis, thawed at room temperature (RT) and then homogenized. An amount, approximately 0.5 g, of each honey sample was transferred into a Teflon vessel with 5 ml nitric acid (67% TraceMetal Grade, Fisher Scientific, Loughborough, UK) and 1.5 ml hydrogen peroxide (30% analytical grade, Sigma-Aldrich, St. Louis, MO, USA) for microwave digestion. The microwave (Start D, Milestone, Sorisole, Italy) programme consisted of three steps: 5 min from RT to 180°C, 10 min hold at 180°C, and 20 min vent. After cooling, the digested sample solutions were quantitatively transferred into disposable flasks and diluted to 100 ml with deionized water produced by a water purification system (Purelab DV35, ELGA, High Wycombe, Buckinghamshire, UK). Analysis of the following elements: Fe, Zn, Cu, Cd, Pb, and As, was performed by inductively coupled plasma mass spectrometry (ICP-MS) (iCap Q mass spectrometer, Thermo Scientific, Bremen, Germany). The most abundant isotopes were used for quantification. The statistical analysis was performed using the GraphPad Prism version 7.00 software. The concentrations of heavy metals in multifloral honey types were expressed as the minimum, maximum, and mean  $\pm$  standard deviation (SD) and were subjected to analysis of variance (One-way ANOVA). The parameters were analyzed using the Student's t-test at the probability of 0.05. Pearson's correlation analysis was applied to examine the relationship between heavy metal concentrations.

## 3. Results and Discussion

Findings of the six examined trace elements are given in Table 1.

**Table 1.** Mean concentrations of selected metals in 92 Serbian multifloral honeys (mg/kg)

Element	Statistics	Multifloral honey
As	Mean $\pm$ SD	0.003 $\pm$ 0.001
	Range (min-max)	0.001-0.004
Cu	Mean $\pm$ SD	0.31 $\pm$ 0.21
	Range (min-max)	0.09-0.92
Zn	Mean $\pm$ SD	1.95 $\pm$ 1.70
	Range (min-max)	0.37-8.02
Fe	Mean $\pm$ SD	2.21 $\pm$ 1.00
	Range (min-max)	0.77-3.94
Cd	Mean $\pm$ SD	0.003 $\pm$ 0.001
	Range (min-max)	0.001-0.01
Pb	Mean $\pm$ SD	0.005 $\pm$ 0.001
	Range (min-max)	0.004-0.026

As, Cu, Zn, concentrations were greater compared to previous studies of multifloral honey samples, while Pb, Cd and Fe were lower [10]. The range of element concentrations for different honeys was greater for

Cu and Pb, lower for Zn, Cd and Fe, and about the same for As. Comparable results were obtained in Turkey for multifloral honeys: Zn (0.65-3.2 mg/kg), Fe (1.55-12.9 mg/kg), Cu (9.97-29.5 mg/kg), Cd (0.29-2.03 mg/kg), Pb (1.54-36.7 mg/kg) (11). Multifloral honey from Croatia had similar content of Cd, but greater concentrations of As, Cu and Pb (12).

Some pairs or even multiple elements can be found in natural matrices together, meaning that it is possible to determine correlations between them, in the same sample matrix, or even in different, sequential matrices, e.g. soil, plant nectar and honey. Correlations between the tested elements are presented in Table 2.

**Table 2.** Matrix of correlation coefficients (r)

Element	As	Cu	Zn	Fe	Cd	Pb
As	1	-0.099	0.252	0.383	-0.133	-1.00
Cu		1	-0.117	0.418	0.426	-0.107
Zn			1	-0.079	-0.100	-0.216
Fe				1	0.064	-0.391
Cd					1	0.268
Pb						1

Regarding the honey samples, there were positive correlations between all metals except Fe and Zn, since these two elements were negatively correlated. There were positive correlations between Pb and Ni, Pb and Cd, and Pb and Fe content in wax. There were strong positive correlations between Ni and Cd, Ni and Fe, and Ni and Mg. Cd content was positively correlated with contents of tested metals in wax and propolis [7].

Serbian national regulation [13] defines criteria for trace elements in honey, Table 3. The table also contains values of method performance for elements, showed as limit of detection (LOD).

**Table 3.** Maximum residue limits (MRLs) for heavy metal content [13] and limits of detection of the ICP-MS method for the tested elements

Element	MRL (mg/kg)	LOD (mg/kg)
As	0.5	0.001
Cu	1	0.02
Zn	10	0.12
Fe	20	0.08
Cd	0.03	0.001
Pb	0.1	0.002

The results obtained during this study show that the honeys' element concentrations were less than the maximum residue limits (MRL).

#### 4. Conclusion

The most abundant element, on average, was Fe, with a mean concentration of 2.21mg/kg, followed by (mg/kg) Zn (1.95), Cu (0.31), Pb (0.005), As (0.003) and Cd (0.003). The widest range of concentrations (mg/kg) of the examined elements in the honeys was for Zn (0.37-8.02), then Fe (0.77-3.94), Cu (0.09-0.92), Pb (0.004-0.026), Cd (0.001-0.01) and As (0.001-0.004). The results obtained showed some positive correlations: Zn-As, Fe-As, Fe-Cu, Fe-Cd, Cd-Cu and Cd-Pb. The strongest negative correlation was noticeable between Pb and As ( $r = -1$ ), followed by correlations of Pb and Fe ( $r = -0,391$ ), Pb and Zn ( $r = -0,216$ ), Cd and As ( $r = -0,133$ ), Zn and Cu ( $r = -0,117$ ), Pb and Cu ( $r = -0,107$ ), Cd and Zn ( $r = -0,100$ ) and finally Cu and As ( $r = -0,099$ ).

#### Acknowledgment

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# Evaluation of phosphate replacement with natural alternatives in chicken patties as a novel approach

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**Abstract.** Polyphosphates are known to increase both the amount of bound water and the strength of the meat particle-particle binding in processed meat products. However, several health risks related to dietary phosphate intake are driving the meat industry to improve product formulations (less phosphate) and to search for alternative phosphate replacers. The aim of this research was to investigate the effects of using eggshell powder as a phosphate replacer on some quality characteristics of chicken patties. Chicken patties were subjected to four treatments, as follows: control contained 0.5% sodium tripolyphosphate; 0.5% eggshell powder; 0.5% eggshell powder+0.25% pectin, and; 0.5% eggshell powder+0.25% carrageenan. Chemical composition, technological parameters and sensory properties were evaluated in all patties. Total moisture, protein, fat and ash in uncooked/cooked patties were in the range of 72.20-75.24%, 13.84-15.39%, 8.14-10.87% and 2.71-3.14%, and 68.59-72.33%, 15.11-18.12% and 8.36-10.99%, respectively. The patties with 0.5% eggshell powder+0.25% carrageenan had the highest water holding capacity and cooking efficiency percentage in relation to pH among the patties studied. The results show the combination of eggshell powder with pectin or carrageenan could be an alternative additive for phosphate-free meat products.

## 1. Introduction

Phosphates are multi-functional, low-cost compounds that enhance product yield by increasing water holding capacity, improve color, flavor, and texture, and have antioxidant functions. Other beneficial effects of phosphates are; stabilization of emulsions and improved texture of meat products by increasing the extraction of salt-soluble proteins based on increasing ionic strength and charges, and reduction of lipid oxidation through their metal chelating activity, which subsequently inhibits off-flavor development <sup>[1]</sup>.

Phosphates are chemical synthetic analogues. Research shows that high dietary phosphate intake increases the risk of kidney and bone diseases, as well as triggering potential cardiovascular and pulmonary diseases. According to recent research, several health risks are caused by phosphate salts, and therefore, reducing the amount of phosphates in product formulations or replacing them with natural components that can provide the same technological effects have come to the fore. Although the negative effects of high salt consumption on health have been known for a long time, studies on the possible health risks caused by the use of phosphate above normal levels have been conducted, in general, since the beginning of the 2000s. Many previous studies have attempted to improve the functionalities of meat products by using various functional ingredients, such as inulin, pectin <sup>[2]</sup>, functional carbohydrates, including guar gum, carrageenan, alginic acid and chitosan <sup>[3]</sup>, 0.2% oyster shell calcium powder, 0.3% egg shell calcium powder, and 0.25% whey protein concentrate <sup>[4]</sup>. Natural calcium powders, which are widely used in the meat industry, include oyster shell calcium (OSC), egg shell calcium (ESC), marine algae calcium (MAC), and whey calcium (milk calcium, MC).



Each of these natural calcium powders has their own unique physico-chemical properties and sensory characteristics, as they differ in their basic sources from raw materials and manufacturing methods. Because of these differences, they provide different processing properties when added to meat products [5]. The effects of different combinations of premixed natural calcium powders on the quality properties of cooked pork products were investigated, with the aim of developing high-quality phosphate-free meat products [6]. Eggshell primarily contains calcium, magnesium carbonate (lime) and protein [7], so eggshell powder (ESP) can be an attractive source of calcium in human nutrition.

Another phosphate replacer, pectin, is used as coating, emulsifier, stabilizer and gelling agent in meat products. Studies show pectin should be used with other additives like calcium to form a good gel suitable for use as a natural phosphate replacer. As a result, pectin is useful in meat products but should not be used alone. Instead, different substances should be used together with pectin in order to provide the desired features of a phosphate replacer [8].

Carrageenan is composed of sulfated linear polysaccharides of D galactose and 3,6-anhydro D galactose, and is extracted from red sea algae. It has no nutritional value, is used by the food industry due to its gelling, thickening and stabilizing properties, and recently, has been used in reduced fat meat products [9]. When carrageenan is incorporated in low fat meat products, it improves the textural characteristics of the final product by decreasing toughness and increasing juiciness [10].

To the best of our knowledge, in previous studies, the suitability of eggshell powder in combination with pectin or carrageenan as natural phosphate replacers in chicken patties has not been studied. Consequently, the aim of this study was to evaluate the effect of the use of eggshell powder alone and with pectin or carrageenan as natural phosphate replacers on some quality characteristics of chicken patties.

## 2. Materials and Methods

### 2.1. Raw material

Chicken breast and thigh meat was obtained from a local market and stored at 4 °C prior to production. All subcutaneous fat and intermuscular fat were removed. Food grade sodium tripolyphosphate (STPP) was kindly donated by Pacovis Food Co. (Izmir, Turkey), pectin was supplied by Sigma-Aldrich Co. (Istanbul, Turkey), carrageenan was purchased from Smart Chemical Co. (Izmir, Turkey) and chicken skin was purchased from a local market. Eggshell powder was prepared in laboratory.

### 2.2. Experimental design and preparation of chicken patties

Four different chicken patties were formulated; in control patties (C), 0.5 g/100 g food grade sodium tripolyphosphate was added. Other patties were formulated with: 0.5% eggshell powder (E); 0.5 % E+0.25% pectin (EP), and; 0.5% E+0.25% carrageenan (EC) as phosphate replacers. Salt (1.5%) and ice (15%) was added to all formulations. Chicken skin was used (15%) as the fat source. Chicken meat (breast and thigh) and skin were ground through a 3 mm plate grinder (Arnica, Turkey), separately. Batches (approximately 700 grams) of each formulation were mixed with a thermomixer (Thermomix, Germany) until a homogenous mixture was obtained (3 min), then mixtures were processed into chicken patties by using a metal shaper. Patties were cooked in an electric oven (Teba, Turkey) at 180°C until the core temperature reached 73°C. Patties were cooled to room temperature and analyses were performed.

**Table 1.** Formulations of chicken patties

Patty formulation	Meat (g)	Chicken skin (g)	Ice (g)	NaCl (g)	NaNO <sub>2</sub> (g)	STPP (g)	ESP (g)	Pectin (g)	Carrageenan (g)
C	68.35	15	15	1.5	0.05	0.5	0	0	0

E	67.95	15	15	1.5	0.05	0	0.5	0	0
EP	67.70	15	15	1.5	0.05	0	0.5	0.25	0
EC	67.70	15	15	1.5	0.05	0	0.5	0	0.25

C: 0.5% sodium tripolyphosphate, E: 0.5% eggshell powder, EP: E+0.25% pectin and EC: E+0.25% carrageenan

Moisture and ash contents of the uncooked and cooked patties were determined according to the AOAC procedure (2012). Protein content of the patties was determined using an automatic nitrogen analyzer (FP 528 LECO, USA) based on the Dumas method. Fat content was evaluated according to Flynn and Bramblet (1975). pH of the patties was measured in triplicate using a pH-meter (WTW pH 3110 set 2, Germany), equipped with a penetration probe.

The percent cooking yield was determined by calculating weight differences for patties before and after cooking <sup>[11]</sup>. Water holding capacity was determined according to Hughes, Cofrades, and Troy (1997) with slight modifications <sup>[12]</sup>. The moisture retention was determined according El-Magoli et al. (1996) <sup>[13]</sup>. Fat retention was calculated according to Murphy et al. (1975) <sup>[11]</sup>. The Bradford method was used to measure protein solubility. Patties were imaged by scanning electron microscopy (SEM) (Thermo Scientific Apreo S, USA) after dried specimens of patties were sputter coated with gold (Leica EM ACE600, Germany). The Apreo SEM benefits from the unique in-lens backscatter detection, which provides excellent materials contrast, even at tilt, short working distance, or on sensitive samples.

Patties were randomly assigned for sensory evaluation. Patties were served warm to a ten-membered panel (graduate students and staff of Ege University Food Engineering Department). At each session, three patty samples were served immediately to panelists and were subjected to sensory evaluation for appearance, color, texture, flavor, juiciness, and overall acceptability.

The experiment was performed twice and the data was evaluated by two-way analysis of variance (ANOVA) using SPSS software version 21.0. Differences among the means were compared using Duncan's Multiple Range Test.

### 3. Results and Discussion

Chemical composition and pH of uncooked and cooked patties are presented in Table 2. Total moisture, protein, fat and ash contents in uncooked patties were between 72.20-75.24%, 13.84-15.39%, 8.14-10.87% and 2.71-3.14%, respectively. The differences of formulation resulted significant changes in moisture, protein and fat contents of uncooked patties ( $P < 0.05$ ) while no effect was recorded on ash content ( $P > 0.05$ ). Uncooked EC patties had the highest moisture content among the patty groups ( $P < 0.05$ ). Uncooked EP patties had a similar protein content as C patties ( $P < 0.05$ ). The highest fat content was measured in EP patties, both uncooked and cooked. The pH of uncooked patties was between 6.16 and 6.22. Total moisture, protein and fat contents in cooked patties ranged between 68.59-72.33%, 15.11-18.12% and 8.36-10.99% respectively. The pH of cooked patties ranged from 6.18 to 6.27. Cooking resulted in increased pH, protein, fat and ash contents of patties, while moisture content decreased due to cooking loss. EC patties had the highest moisture content ( $P < 0.05$ ), so adding carrageenan led to an increase in total moisture content. This finding could be the result of the high WHC of carrageenan. The uncooked patty mixes of C and EC had higher pHs than other mixtures ( $P < 0.05$ ), which could be the result of the greater effectiveness of EC in altering the pH. Adding eggshell powder with pectin and carrageenan increased the pH of cooked patties, and the highest pH was measured in cooked EP and EC patties ( $P < 0.05$ ). Among cooked patties, E patties had the lowest pH ( $P < 0.05$ ). These results showed that eggshell powder alone was not suitable phosphate replacer, so this product should be combined with other materials to produce more acceptable phosphate-free meat products.

**Table 2.** Chemical composition of uncooked and cooked patties

	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	pH
Uncooked patties					
C	72.20±0.15 <sup>c</sup>	15.39±0.55 <sup>a</sup>	9.43±0.37 <sup>b</sup>	3.14±0.31 <sup>a</sup>	6.20±0.01 <sup>a</sup>
E	73.58±0.56 <sup>b</sup>	13.84±0.35 <sup>b</sup>	8.14±0.64 <sup>c</sup>	2.71±0.09 <sup>b</sup>	6.17±0.00 <sup>b</sup>
EP	73.39±0.86 <sup>b</sup>	15.16±0.53 <sup>a</sup>	10.87±0.33 <sup>a</sup>	2.71±0.16 <sup>b</sup>	6.16±0.00 <sup>b</sup>
EC	75.24±0.39 <sup>a</sup>	14.21±0.35 <sup>b</sup>	10.08±0.85 <sup>ab</sup>	2.80±0.24 <sup>ab</sup>	6.22±0.01 <sup>a</sup>
Cooked patties					
C	68.59±1.87 <sup>b</sup>	18.12±0.66 <sup>a</sup>	9.99±0.73 <sup>a</sup>	3.75±0.16 <sup>a</sup>	6.23±0.01 <sup>b</sup>
E	70.72±0.61 <sup>ab</sup>	17.05±0.60 <sup>a</sup>	8.36±1.07 <sup>b</sup>	3.13±0.10 <sup>b</sup>	6.18±0.01 <sup>c</sup>
EP	72.33±1.06 <sup>a</sup>	16.68±1.02 <sup>ab</sup>	10.99±0.13 <sup>a</sup>	2.98±0.00 <sup>b</sup>	6.27±0.01 <sup>a</sup>
EC	69.53±0.72 <sup>b</sup>	15.11±0.87 <sup>b</sup>	9.96±0.15 <sup>a</sup>	3.15±0.01 <sup>b</sup>	6.27±0.01 <sup>a</sup>

<sup>a-d</sup> Mean±SD with different superscript letters indicate significant differences (p<0.05)

C: 0.5% sodium tripolyphosphate, E: 0.5% eggshell powder, EP: E+0.25% pectin and EC: E+0.25% carrageenan

Cooking characteristics and protein solubility values are shown in Table 3. Cooking yield was between 79.49%-85.91%, and the lowest cooking yield was recorded in EP patties. WHC was between 72.19-82.28, depending on pH. Moisture retention values were between 66.52-62.71%, and the highest moisture retention was observed in EP patties, while the lowest was observed in EC patties. Moisture retention in meat products is an important cooking parameter, since retained moisture in the product affects eating quality [14]. The fat retention percentages were between 85.29-96.06%. Fat retention was the greatest in C and EC patties, formulated with sodium tripolyphosphate and carrageenan, respectively. The protein solubility was significantly affected by patty formulation and was the highest (1118.01 µg protein/ml) in EC patties, using eggshell powder along with carrageenan.

**Table 3.** Cooking characteristics and protein solubility of chicken patties

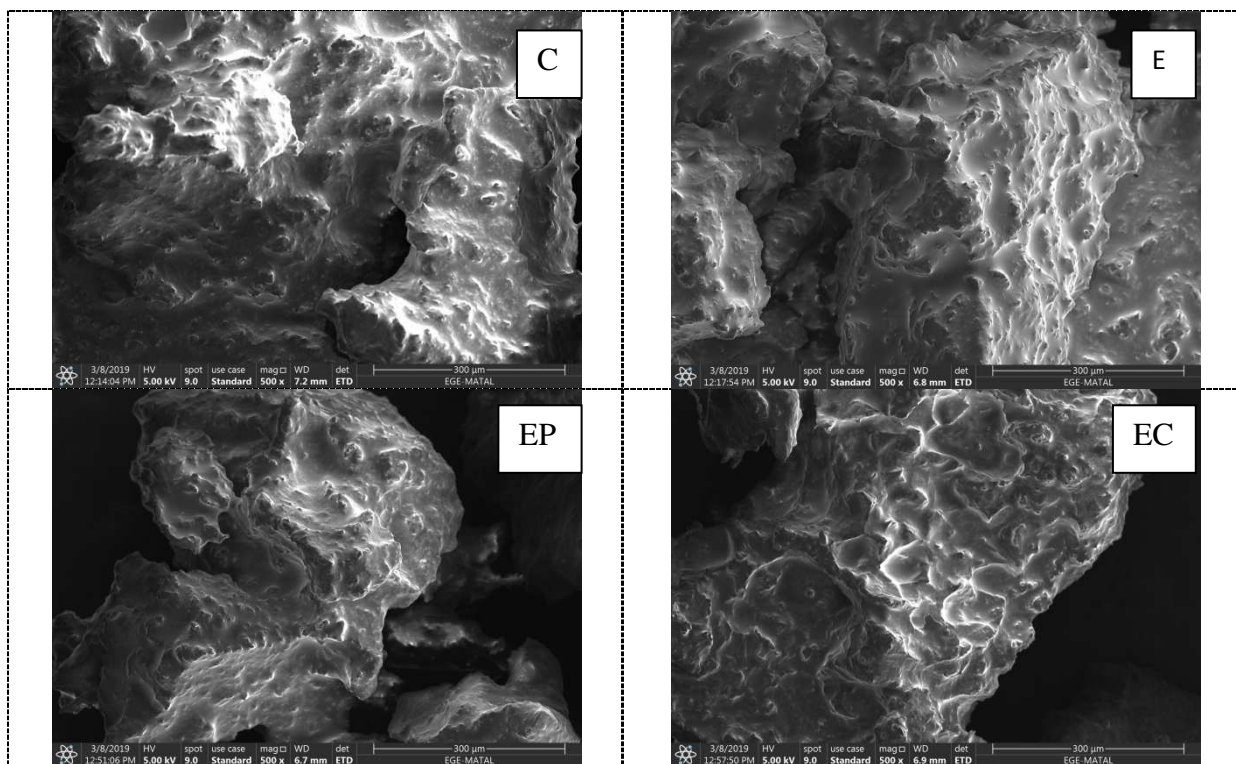
	Cooking Yield (%)	WHC	Moisture Retention (%)	Fat Retention (%)	Protein Solubility (µg protein/ml)
C	85.12±0.35 <sup>b</sup>	75.82±0.96 <sup>b</sup>	63.95±1.64 <sup>bc</sup>	96.06±1.82 <sup>a</sup>	962.40±3.39 <sup>c</sup>
E	83.86±0.57 <sup>c</sup>	73.52±0.59 <sup>c</sup>	65.27±1.04 <sup>ab</sup>	85.29±1.57 <sup>c</sup>	805.57±7.60 <sup>d</sup>
EP	79.49±0.19 <sup>d</sup>	72.19±0.91 <sup>c</sup>	66.52±0.73 <sup>a</sup>	90.44±1.88 <sup>b</sup>	1026.83±3.07 <sup>b</sup>
EC	85.91±0.27 <sup>a</sup>	82.28±0.49 <sup>a</sup>	62.71±0.56 <sup>c</sup>	94.61±1.49 <sup>a</sup>	1118.01±2.42 <sup>a</sup>

WHC – water holding capacity; <sup>a-d</sup> Mean±SD with different superscript letters indicate significant differences (p<0.05); C: 0.5% sodium tripolyphosphate, E: 0.5% eggshell powder, EP: E+0.25% pectin and EC: E+0.25% carrageenan

Typical SEM micrographs of chicken patties are presented in Figure 1. The distributions of particles and voids in the product structure were seen, enabling us to comment on the quality parameters such as



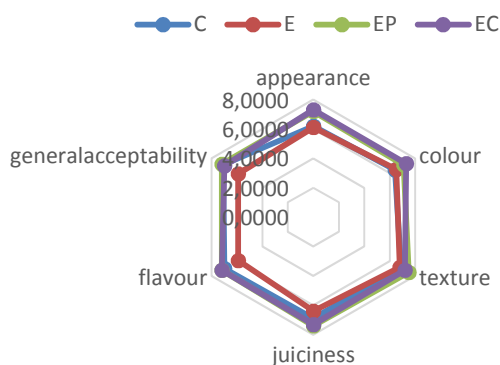
water holding capacity and texture <sup>[15]</sup>. Microstructural differences were observed between the patty formulations. In the C and EC patties particularly, fat globules were retained in the protein network.



C: 0.5% sodium tripolyphosphate, E: 0.5% eggshell powder, EP: E+0.25% pectin and EC: E+0.25% carrageenan

**Figure 1.** Scanning electron microscope images of chicken patties (500 ×)

The results of sensory analysis are presented in Figure 2. Appearance, color, texture, juiciness, flavor and general acceptability of the patties were scored between 6.10-7.30, 6.40-7.30, 6.80-7.50, 6.40-7.40, 5.90-7.20 and 5.90-7.20, respectively. The sensory quality of all patties was acceptable. There was no significant difference in terms of general acceptability between the different patties ( $P>0.05$ ), and therefore, use of eggshell powder provided equivalent sensory characteristics to phosphate. The EP patties received the highest sensory analysis scores.



**Figure 2.** Sensory scores of chicken patties

#### 4. Conclusion

Our study showed that eggshell powder combined with carrageenan would be a suitable natural phosphate replacer in chicken patties. The pH of the patties with the combination of eggshell powder and carrageenan was significantly higher than the other patty types. In all patty groups, the water holding capacity was correlated with cooking yield. The results of our study showed the combinations of eggshell powder with pectin or carrageenan in chicken patties have good potential to enhance these products' chemical, technological and sensory qualities and offers a novel possibility for phosphate replacement in formulation of healthier meat products.

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# Profile of dry sausages traditionally prepared in Pirot, eastern Serbia

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**Abstract.** The aims of this study were to determine the nutritional composition (moisture, protein and total fat) of *peglana* sausages produced in eastern Serbia and to analyze the composition of fatty acids. Determination of fatty acid composition in the sausages was performed after ripening and after 20 days of storage. Also, a sample preparation method for fatty acid analysis after simultaneous microwave-assisted extraction-esterification was implemented and results were compared with conventional extraction. The results obtained show *peglana* sausages have high contents of proteins and saturated fatty acids, but no nitrite; the lack of nitrite makes these sausages a suitable product for consumers trying to avoid this additive. The good agreement between results provided by both fat extraction methods demonstrates the usefulness of both methods as routine methods for the treatment of meat samples prior to fatty acid analysis.

## 1. Introduction

Meat contains micronutrients and all the essential amino acids and minerals, including selenium, vitamin B6 and B12, and vitamin D [1]. *Peglana* sausage is a popular, traditional specialty in Pirot, Serbia, and which has always been a highly appreciated product among the local people because it is prepared from selected top quality meat. Since thermal treatment and smoke are replaced by air drying and an optimum temperature of -5 °C to 5 °C, free from moisture and frost, then it can rightly be said that *peglana* sausage is truly an ecologically friendly product. It is prepared from beef, goat and sheep meat, but pork is never used, because it contributes to quick deterioration of the sausage. An important principle of the traditional production practice is that producers use a minimum of chemical substances during processing. In terms of household production, they do not use additives and preservatives. The production of this traditional product is not standardized and product is generally monitored subjectively without strict control of the characteristics required for *peglana* sausages. However, if hygienic conditions and the intrinsic properties of foods are maintained, these traditional sausages would be considered safe products.

Fats and fatty acids play an important role in giving specific tastes to the different meat species, which is the result of differences in fatty acid profiles in the different animal species [2]. Fatty acids contained in phospholipids are more unsaturated than those found in triacylglycerols. Thus, phospholipids contain relatively large amounts of linolenic and arachidic acids. In recent years, much attention has been paid to developing meat and meat products with physiological functions to promote health conditions and prevent the risk of diseases [3]. Precise and accurate quantification of the fatty acid profile from meat products is extremely challenging, and relies in a multistep process: adequate storage, meaning fatty acids are well preserved; lipid extraction; derivatization of fatty acids; identification and quantification of the derivatized molecules. Ruminant products are characterized by higher content of saturated fatty acids (SFA) and moderate to lower concentration of monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) [4]. In general, the analytical procedure for the determination of fatty acids from meat products comprises three steps: extraction of the fats, esterification and gas chromatographic analysis.

Microwave-assisted extraction is a relatively novel method of extracting soluble products from a wide range of solid materials into a liquid state using microwave energy. Recently, much attention has been given to the application of microwave dielectric heating in analytical chemistry because of the



reduced analysis time, simplified manipulation and higher purity of the final products. Several classes of compounds, such as fats and oils [5], polycyclic aromatic hydrocarbons [6] and metals [7], have been efficiently extracted from a variety of matrices.

## 2. Materials and methods

*Peglana* dry sausages were prepared from goat and beef meat. Moisture content in the sausages was determined in triplicate by the AOAC (1991) method [8], and expressed as a percentage of the sample weight. Protein content was determined by the Kjeldahl method (Total N  $\times$  6.25) [9]. The nitrite content was determined by the spectrophotometric method [10]. Fat was extracted according to the Soxhlet extraction method [11] and using microwave-assisted extraction, and after extractions, the lipid content of the extracts was gravimetrically determined. Five separate replicates of each fat extraction method were prepared on different days and were used to determine the percentage of fatty acids in the total fat content of the sausages.

Fat and fatty acids were extracted using Soxhlet extraction from dry sausages by the hydrolytic method. Fat was extracted into petroleum, and then methylated to fatty acid methyl esters (FAMES). An aliquot of lipid extract (50 mg) was dissolved in 2.4 mL of n-hexane. A methanolic solution (600  $\mu$ L) of 2 M sodium methoxide was then added. The mixture was stirred at room temperature for 2 min, then was acidified with a methanolic solution of 1M HCl and extracted with n-hexane (3 mL). FAMES were quantitatively measured by gas chromatography (GC). The analyses were performed using an Clarus 680 PerkinElmer equipped with a flame ionization detector. The temperature of the column at the beginning was 80 °C and ramped up at 0.5 °C/min, then 4 °C/min to 220 °C, held for 4 minutes, then ramped up at 4 °C/min to 240 °C and held at 240 °C for 10 minutes. The total run time was 56.5 mins. The temperature of both the injection port and the detector was 240 °C. Fatty acids were identified by comparison of their retention times with those of authentic standards (FAME Reference Standard, FAMQ-005, AccuStandard, USA) and reported as percentage of the total fatty acids determined. C19:0 was used as an internal standard. Results were expressed as saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acid (PUFA). The separation of the 37-component FAME standard mixture was performed on a 30 m $\times$ 0.25 mm ID, 0.25  $\mu$ m df PerkinElmer Elite-WAX GC column. The FAMES were quantified as a percentage of total methyl esters. Fatty acids were reported as a percentage of total fatty acids determined.

Microwave assisted extraction was carried out using the Start-E microwave extraction system (Milestone, Sorisole, Italy). Microwave assisted extraction conditions were 500 mg of sample, 10 mL of solvent (hexane:methanol 3:1, v/v), microwave extraction during 10 min at 50 °C and 400 W.

## 3. Results and discussion

The chemical composition of the dry *peglana* sausages studied is shown in Table 1. The *peglana* sausages had high contents of protein (33.0% wet weight) and fat (30.0%). The nutritional value of meat is mainly due to the protein content, which differs according to the location of the muscle in the animal body.

**Table 1.** Chemical composition of dry *peglana* sausages (mean $\pm$ standard deviation)

Parameter	Conventional preparation	Microwave-assisted extraction-esterification
Moisture, %	27.55 $\pm$ 0.5	
Fat, %	30.7 $\pm$ 0.8	30.0 $\pm$ 0.9
Proteins, %	33.0 $\pm$ 0.8	
Nitrite, mg/kg	<0.30	

Table 2 shows the fatty acid composition of *peglana* sausages. FAME analysis was used to characterize the lipid fraction in dry *peglana* sausages. The fatty acid composition of fat is a complex

mixture of SFAs, MUFAs and PUFAs with various carbon chain lengths. The 37-component FAME standard mix is designed to mimic the fatty acid composition of many food samples, and it can be used to identify key FAMES derived from fatty acids in meat products. This mix contains FAMES ranging from C4:0 to C24:1, including most of the important saturated, monounsaturated, and polyunsaturated FAMES. FAMES were identified by comparing the retention times of the chromatographic peaks with those of the FAME standard.

MUFAs were the major constituents of the fat content in the sausages, ranging from 54.72% to 56.35% of the total fat. Octadecenoic (oleic) acid (C18:1), the predominant MUFA, was found in high amounts, ranging from 31.25% to 31.97%. Palmitic acid (16:0) was the major SFA found, ranging from 23.44% to 24.32%. Good separation was obtained between the fatty acids, except for one pair of C18:1 *cis* and C18:1 *trans* isomers, the peaks of which were obscured.

There is evidence that the type of fat is more important than the total amount of fat in the quantification of cardiovascular disease risk. The analysis of fatty acids has become increasingly important in a modern society with dietary recommendations favoring a low intake of fats. Consequently, there is growing interest in monitoring the composition of fatty acids in sausage and determining the PUFA/SFA ratio [12].

Because water has a major functional and quality impact on processed meats, there are numerous regulations controlling the addition and/or final water content of processed meats. The ionic strength of the salt-water phase, for example, is necessary for the solubilization and extraction of the myofibrillar proteins which are responsible for stabilizing fat in emulsion products, binding of muscle pieces in restructured products and producing product textural properties that result from heat-set gelation of the proteins. Oxidative processes in fermented meat products lead to the degradation of unsaturated fatty acids, cholesterol and proteins (including pigments), although the presence of sodium chloride can have an accelerating effect on lipid oxidation [13]. Table 1 shows a small amount of water in our *peglana* sausages, so the drying process was suitable according to the dry matter and the low content of unsaturated fatty acids (Table 2). Meat contains a high percentage of water. The majority of water in muscle is held within the structure of the muscle itself or within myofibril. Water can be divided into three types in muscle, i.e., bound, entrapped (immobilized) or free water. The content of bound water held closely to protein is a very small portion of the total water in muscle cells. Therefore, water significantly affects the structure and quality of meat, not only after slaughter but also during the storage time. In addition, water affects the sensory and textural properties of meat products, since water is a good solvent for the reactions occurring inside the meat, and is a suitable environment for growth of microorganisms.

Goat meat has a fatty acid profile that is beneficial to consumer health due to the high concentrations of oleic acid, the presence of essential fatty acids, and low concentrations of lauric, myristic, and palmitic acids when compared to the meats of other species. The greater amounts of oleic acid (C18:1) in goat meat could be attributed to the greater animal biosynthesis from stearic acid (C18:0) [14]. Therefore, nutritional strategies that increase the conversion of stearic acid into oleic acid would contribute to an improvement in meat quality.

**Table 2.** Fatty acid profile (% of total fatty acids; mean±standard deviation) of sausages (n=5) using conventional and microwave-assisted extraction-esterification (ME)

<i>Fatty acid</i>		<i>Conventional preparation</i>	<i>Microwave-assisted extraction-esterification</i>
Myristic acid	C14:0	1.56±0.05	1.50±0.04
Myristoleic acid	C14:1	0.15±0.03	<0.05
Pentadecanoic acid	C15:0	0.43±0.05	0.38±0.06
cis-10-Pentadecanoic acid	C15:1	0.13±0.03	<0.05

Palmitic acid	C16:0	24.32±0.10	23.44±0.10
Palmitoleic acid	C16:1	1.07±0.04	1.04±0.03
Heptadecanoic acid	C17:0	1.05±0.04	1.02±0.07
cis-10-Heptadecanoic acid	C17:1	0.27±0.03	0.22±0.02
Stearic acid	C18:0	28.99±0.5	28.38±0.5
Oleic acid	C18:1 cis(n9)	31.97±0.6	31.25±0.6
Elaidic acid	C18:1 trans(n9)	4.20±0.06	4.11±0.07
Linoleic acid	C18:2 cis (n6)	3.49±0.06	3.20±0.11
Linolelaidic acid	C18:2 trans (n6)	0.21±0.03	0.10±0.03
γ-Linolenic acid	C18:3n6	0.15±0.02	0.13±0.02
α-Linolenic acid	C18:3n3	0.41±0.03	0.36±0.09
Erucic acid	C22:1(n9)	0.87±0.10	1.80±0.09
cis-13,16-Docosadienoic acid	C22:2(n6)	0.72±0.20	3.07±0.10
	ΣSFA	56.35	54.72
	ΣMUFA	38.66	38.42
	ΣPUFA	4.98	6.86
	Total n-6	4.57	6.50
	Total n-3	0.41	0.36
	Total n-9	37.04	37.16

The results showed that microwave-assisted extraction can be used to study lipids from meat samples without the risk of chemical changes during the extraction process. After microwave-assisted extraction, there was no difference in fatty acid content between the conventional method and sample preparation with microwave-assisted extraction, with the one exception of cis-13,16-Docosadienoic acid content. There were several long-chain n-6 PUFAs in the sausages, but unfortunately, there was only a small amount of long-chain total n-3 PUFAs in the sausages. The n-3 PUFAs are not only essential nutrients for humans, but also are significant in helping protect consumers from inflammatory disease, diabetes, some cancers and behavioral disorders. Therefore, increasing n-3 PUFA in meat contributes to improving consumer health, and would help to combat the negative image of ruminant meat [10].

The PUFA/SFA ratio is one of the major parameters currently used to assess the nutritional quality of the lipid fraction of foods. The recommended PUFA/SFA ratio in human diets is >0.4 [16], but in our study, the PUFA/SFA ratios of *peglana* sausage were unsatisfactorily low, 0.087 and 0.125 using conventional and microwave-assisted extraction methods, respectively.

SFA are considered to raise plasma cholesterol, except for stearic acid which reduces total and low density lipoprotein cholesterol; therefore, the content of stearic acid is subtracted from the SFA fraction when the association between food saturated fatty acids and risk of heart diseases is studied. Moreover, MUFA have a hypocholesterolemic effect, but they do not decrease high density lipoprotein cholesterol, which protects against cardiovascular diseases. It is also possible to find trans fatty acids in beef as they are formed as a result of the biohydrogenation by rumen bacteria.

As the product tested was without additives, the nitrate content was below the limit of quantification (Table 1). Nitrite can cause the formation of carcinogenic N-nitrosamines in cured meat products owing to nitrite's reaction with secondary amines and amino acids in muscle proteins [16].

Microwave-assisted extraction of fat could become the method of choice because of its precision, accuracy and speed. Other advantages of microwave-assisted extraction of fat are the reduction of consumption of organic solvents and the shorter time for analysis than is required using the conventional extraction procedure.

#### 4. Conclusion

The aim of the present research was to implement a sensitive and efficient method for simultaneous extraction and determination of fatty acids from dry sausages. The combination of microwave-assisted extraction with esterification was successfully applied to rapid isolation and pre-concentration of the target analytes prior to analysis by gas chromatography-flame ionization detection. The fatty acid composition measured in *peglana* sausages, and obtained by using the simultaneous microwave-assisted extraction-esterification method and the conventional Soxhlet extraction-esterification method, can be regarded as equivalent.

The presented results show that *peglana* sausage has a desirable chemical composition, high percentages of proteins and fats, but an undesirably high PUFA content. These sausages are typically produced in the eastern Serbia and are a significant part of the culinary traditions of this region.

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## Chromium content in the meat of male Saanen goat kids from Vojvodina (Northern Serbia)

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**Abstract.** Goats, the earliest ruminant to be domesticated, are traditional sources of meat, milk, fibre, leather, related products of animal origin and as draught and pack animals. Meat is the major product of the goat. Meat quality is the sum of all sensory, nutritive, technological and hygienic-toxicological factors of meat.

The aims of this study were to investigate the chromium content of four different muscles (*M. psoas major*, *M. longissimus dorsi*, *M. semimembranosus* and *M. triceps brachii*) of Saanen goat male kids and to determine whether the chromium contents differed between the muscles. Chromium content was determined using inductively coupled plasma optical emission spectrometry (ICP-OES), after dry ashing mineralisation. The studied muscles did not significantly differ ( $P > 0.05$ ) with respect to chromium content. The chromium content ranged from 0.012 to 0.067 mg/100 g, with an average of 0.026 mg/100 g.

### 1. Introduction

Goats, the earliest ruminant to be domesticated, are traditional sources of meat, milk, fibre, leather, related products of animal origin and are used as draught and pack animals [1,2]. Saanen dairy goats originated from the Saanen valley of Switzerland, and are very famous for their excellent milk production, as well as for being the largest of the dairy breeds [3].

The most important category of goat meat in Serbia, as in developed countries, is kid meat [4]. The greatest demands for young goats in Serbia are seasonally during the springtime. Production is based on various breeds, more or less locally determined. In some regions, production of goats is quite extensive. The number of kids (and goats) that are slaughtered annually in Serbia is not negligible. These, however, are not available on the market, since most kids and adult goats are slaughtered and consumed on the farms where they were raised [4,5].

Traditionally, kids are slaughtered at 3-7 months old and 12-15 kg carcass weight [6]. According to Serbian legislation [7], kids are normally slaughtered between 3 weeks and 6 months of age. The



carcass weight with head, liver, heart, lungs, kidney and internal fat and without skin and distal parts of the legs should be between 4 and 12 kg.

Meat quality is the sum of all sensory, nutritive, technological and hygienic-toxicological factors of meat. The nutritive factors of meat quality include proteins and their composition, fats and their composition, minerals, vitamins, utilisation, digestibility and biological value [8,9]. Minerals are the inorganic elements, other than carbon, hydrogen, oxygen and nitrogen, which remain behind in the ash when food is incinerated. They are usually divided into two groups – macrominerals (main elements) and microminerals (trace elements) or into three groups – main elements (macrominerals), trace elements (microminerals) and ultra-trace elements. Many of the minerals are essential for plants, animals and humans. The main elements (Na, K, Ca, Mg, Cl, P, S) are essential for human beings in amounts >50 mg/day. Trace elements (Fe, I, F, Zn, Se, Cu, Mn, Cr, Mo, Co, Ni) are essential in amounts of <50 mg/day. Ultra-trace elements are: Al, As, Ba, Bi, B, Br, Cd, Cs, Ge, Hg, Li, Pb, Rb, Sb, Si, Sm, Sn, Sr, Tl, Ti and W [10,11]. The chromium (Cr) content of the human body varies considerably depending on the region; the range is 6-12 mg. The daily intake also varies greatly, from 5 to 200 µg. However, this supply is considered suboptimal. Cr is important in the utilisation of glucose. For instance, it activates the enzyme phosphoglucomutase and increases the activity of insulin; therefore, Cr deficiency causes a decrease in glucose tolerance. Additionally, Cr deficiency increases the risk of cardiovascular disease [11].

There is a lack of information about the mineral composition, and especially about the Cr content, of meat from goat kids. Therefore, the aims of this study were to determine the Cr contents of four major muscles (*M. psoas major*, *M. longissimus dorsi*, *M. semimembranosus*, and *M. triceps brachii*) derived from intensively reared male Saanen goat kids, and to determine whether the contents of this trace element differed in the different muscle types.

## 2. Materials and methods

Twenty, from 67 to 83 day-old, male Saanen goat kids (body weight from 19.5 to 23.9 kg) of similar background were used in the experiment. All kids were raised under intensive, identical husbandry; management and feeding conditions and pre-slaughter handling practices were as described in detail in Tomović et al. [12,13].

At the end of the fattening period, all kids were transported to a commercial abattoir. Kids were held overnight without feed before slaughter. Kids were slaughtered and dressed using standard commercial procedures. Carcasses were conventionally chilled for 24 h in chiller at 0-4°C. The cold carcasses were split down the dorsal midline and right sides were used for the present study. The following four muscles were excised from the right side of each carcass: *M. psoas major* (PM), *M. longissimus dorsi* (LD), *M. semimembranosus* (SM) and *M. triceps brachii* (TB). The meat samples were trimmed of visible adipose and connective tissue.

After trimming, each muscle was homogenised (Waring 8010ES Blender, USA; capacity 1 L, speed 18,000 rpm, duration of homogenisation 10 s, temperature after homogenisation <10°C), vacuum packaged in polyethylene bags and stored at -40°C until determination of Cr content.

The Cr content in the muscle tissues was determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (iCP 6000 Series, Thermo Scientific, Cambridge, United Kingdom), after dry ashing mineralisation as described in detail in Tomović et al. [14,15,16] and Jokanović et al. [17].

All data are presented as mean, standard deviation (SD) and range. Analysis of variance (Duncan's multiple range test) was used to test the hypothesis about differences among mean values. The software package STATISTICA 10 [18] was used for analysis.

## 3. Results and discussion

The average content, standard deviation and range of Cr in the investigated samples of the meat tissues from four different muscles are presented in Table 1. The Cr contents found in the present study did not differ significantly ( $P = 0.142$ ) amongst the meat tissue belonging to different muscles of male

Saanen goat kids. The mean Cr content in the PM, LD, SM and TB muscles was 0.033, 0.019, 0.027 and 0.026 mg/100 g, respectively. The average content of Cr in the meat from these male Saanen goat kids in Vojvodina was 0.026 mg/100 g. The minimum Cr content found in the male goat kid meat was 0.012, while the maximum Cr content was 0.067 mg/100 g.

**Table 1.** Chromium content of four muscles from male Saanen goat kids

Muscle		Cr (mg/100 g wet weight)
<i>M. psoas major</i>	Mean $\pm$ SD	0.033 $\pm$ 0.009
	Range	0.022–0.053
<i>M. longissimus dorsi</i>	Mean $\pm$ SD	0.019 $\pm$ 0.009
	Range	0.012–0.042
<i>M. semimembranosus</i>	Mean $\pm$ SD	0.027 $\pm$ 0.020
	Range	0.012–0.067
<i>M. triceps brachii</i>	Mean $\pm$ SD	0.026 $\pm$ 0.010
	Range	0.014–0.048
<i>P</i> value		0.142
All muscles	Mean $\pm$ SD	0.026 $\pm$ 0.013
	Range	0.012–0.067

In the scientific literature there is a lack of information about the Cr content in meat from male goat kids. Generally, the Cr content, in the meat of male Saanen goat kids from Vojvodina, obtained in this study, was somewhat higher than the values reported for the Cr content of other types of red meat (beef, lamb, pork) reported in the Danish Food Composition Database [19].

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# Assessment of meat products and saturated fatty acid intake in human diets

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**Abstract.** The World Health Organization (WHO) has released a guide to proper diet in which the food pyramid is presented. The food pyramid indicates graphically which types of food should be eaten daily. Changes in the diet of any population are slow and difficult to achieve. The aim of this study was to investigate: a) the fatty acid composition of processed meat products, and; b) the daily saturated fatty acid (SFA) content, as calculated by estimated consumption of processed meat products (meat pieces preserved in plastic casing by heat treatment) present in the Serbian market. Consumption of 100 g of preserved pork meat pieces per day equated to 4.64 g saturated fat or 23.20% of daily fat intake being saturated, while consumption of preserved poultry meat pieces would result in 1.73 g of saturated fat, or 8.65% of daily fat intake being saturated which is closer to the recommended daily intake of 10% of fats being saturated. Further research is needed to understand better the optimal combination of unsaturated fatty acids in these processed meat products and recommended daily intakes that should maintain and enhance the health status of consumers.

## 1. Introduction

When planning a diet, the first step is to calculate energy needs. The World Health Organization (WHO) has released a guide to proper diet in which the food pyramid is presented [1]. The food pyramid graphically illustrates which types of food should be eaten daily. A red colour denotes foods that should be consumed rarely. An orange colour indicates foods which should be moderately consumed. A green colour indicates foods that should be eaten most often. The base of the pyramid includes cereals and their products, which should account for 35-45% of the total daily energy intake (marked in green). The next step in the pyramid consists of fruits and vegetables, with fruit accounting for 15% and vegetables for 20% of the total daily energy intake (marked yellow-green). The third step consists of the milk and milk products and groups of meat, fish and eggs both with 10% of the daily energy intake (marked yellow-red). At the top of the pyramid are oils, fats, and sugars, which should account for only 5% of the daily energy intake (marked in red).

Changes in population diets are slow and difficult to achieve. Sudden and radical change in a population's diet can cause distrust and rejection. However, the countries that have set intermediate dietary targets for their populations have experienced good acceptance, and satisfactory changes and health effects, and this approach is supported by the WHO [2]. WHO European Food and Nutrition Action Plan 2015-2020 aims to promote healthy diets by addressing priorities such as excessive intake



of energy, saturated fats and *trans* fats, sugar and salt, and inadequate consumption of vegetables, fruits and whole grains [2].

The prime reason for such concern has been the growing epidemic of overweight resulting from our obesogenic environment with an abundance of cheap and high caloric foods available at any place, any time [3]. A substantial proportion of the population worldwide, including children and adolescents, is now overweight, with far-reaching consequences in terms of increased risk of chronic illness [4]. The overweight epidemic has spurred research into the health consequences of overeating and overweight, and information about this has found its way to the public that now tends to associate eating with health, especially in the United States [5]. WHO recommend that the daily energy intake of fat does not exceed 30%. Almost half of the required amount of fat should originate from monounsaturated fatty acids (MUFAs). It is known that fat combustion produces 9.3 kcal; excessive intake of fat (vegetable and animal sources) increases the energy intake, because high-fat foods with high energy density can lead to obesity and associated pathologies conditions and disorders.

Saturated fatty acids (SFAs) are found predominantly in fat of animal origin (pork, beef and mutton tallow, meat and meat products, milk and milk products), hydrogenated vegetable margarine (solid), and solid vegetable fat. They can increase the levels of harmful LDL and total serum cholesterol, and with that, increase the risk of thrombosis, even though there are proven beneficial effect of stearic fatty acid (C18:0) on human health [6]. Oleic acid (C18:1cis-9) is the most widely distributed MUFA and is predominantly found in olive oil, rapeseed oil, peanut oil and avocado. MUFA maintains the level of protective HDL cholesterol in the blood [6]. Polyunsaturated *n*-3 fatty acids (*n*-3 PUFAs) are found in fish oil (herring, mackerel, sardines, trout) [7]. Regular consumption of these fish twice a week reduces the risk of platelet aggregation during blood clot formation, and reduces the risk of thrombosis, cerebrovascular accident and myocardial infarction [8]. The *n*-3 PUFAs have a small but positive effect on reducing LDL cholesterol and they have a pronounced effect on the lowering of triglycerides in the blood [8]. The *n*-6 PUFAs are found in vegetable oils (sunflower, corn, soybean, safflower oil and cotton oil), and soft margarines. *n*-6 PUFA improves absorption of antioxidant vitamins (vitamins A and E) and other liposoluble vitamins and lower levels of LDL cholesterol. Their daily energy intake is limited to 7%, as increased intake can lead to accumulation of oxidation products [9]. *Trans* fatty acids are formed during hydrogenation of vegetable oils, a product of solid vegetable fats. It was reported that *trans* fatty acids and SFAs have a very similar effects on increasing LDL and decreasing HDL cholesterol levels in the blood [10].

Meat and meat products contain large amounts of SFAs. Pieces or cuts of very fat-rich meat (neck, ribs, sausages, kidneys, knees, paps) are typically cheaper than more expensive cuts and are sources of SFAs [11,12]. As social and economic factors strongly contribute to unhealthy diets and poor nutrition, population-wide strategies, policies and targeted interventions are required by governments [2]. In particular, beneficial effects of the Mediterranean diet have been reported [13,14]. The Mediterranean diet refers to a collection of eating habits traditionally followed by people in the countries bordering the Mediterranean Sea. Mediterranean diet typically consists of high consumption of fruits and vegetables, legumes and complex carbohydrates (whole grains), a moderate consumption of fish, low consumption of red meat, olive oil as the main source of fat, low-to-moderate consumption of red wine, and low-to-moderate consumption of milk and dairy products [13,14].

The aims of this study were to investigate: a) the fatty acid composition of processed meat products, and b) the daily SFA content, as calculated by estimated consumption of processed meat products (heat treated meat pieces) present on the Serbian market.

## 2. Materials and Methods

### 2.1. Samples

Twenty preserved meats were examined, 10 pork and 10 poultry. Heat treated meat products with net weights of 2 kg were purchased from meat companies from Serbia. The finished products were heat

treated in polyamide plastic casing, diameter of 90 mm. The colour, smell and taste of each was characteristic of boiled salted pork or poultry. Pasteurization was conducted until a temperature of 72 °C in the centre of the products was achieved. Ingredients were: category I pork or poultry meat pieces, ice, protein preparations, carbohydrates, spices and stabilizers.

## 2.2 Fatty acid analysis by capillary gas chromatography

The total fat content was determined according to ISO standard method 1443:1973. Total lipids for fatty acid determination were extracted from meat products by accelerated solvent extraction (ASE 200, Dionex, Sunnyvale, CA) according to the method of [15]. Fatty acid methyl esters (FAMES) in the extracted lipids were prepared by transesterification using 0.25 M trimethylsulfonium hydroxide (TMSH) in methanol (EN ISO 5509:2000). FAMES were determined by gas-liquid chromatography (GLC, Shimadzu 2010, Japan) equipped with flame ionization detector and capillary HP-88 column (length 100m, i.d. 0.25 mm, film thickness 0.20 µm). Injector and detector temperature were 250 °C and 280 °C, respectively. Nitrogen was used as the carrier gas at flow rate of 1.33 mL min<sup>-1</sup>. The injector split ratio was set at 1:50. To achieve complete separation of the examined compounds, a programmed column oven temperature starting at 125 °C and ending at 230 °C was applied. Total analysis time was 50.5 min. The chromatographic peaks were identified by comparing relative retention times of FAME peaks with peaks in Supelco 37 Component FAME mix standard (Supelco, Bellefonte, USA). Each sample was analysed in duplicate. Results were expressed as weight of fatty acid (g) in 100 g of fatty acids.

## 2.3. Calculation of daily intake total fat and saturated fats

Percentages of saturated fat derived from preserved meat pieces were calculated in relation to the reference intake of 2,000 kcal. The rules on labelling and advertising in Serbia [16] and in the US [17] recommend an intake of saturated fats of 20 g d<sup>-1</sup>. These values are informative for consumers in interpreting nutritional values of food products. Calculation of saturated fat intakes was performed by dividing saturated fat content expressed in 100 g of product by 20 g.

## 2.4. Statistical analysis

Data obtained for the fatty acid compositions were subjected to analysis of variance (ANOVA) with the Tukey-Kramer HSD test for the comparisons of means at the 5 % level of significance. Statistical analysis was performed using SAS Institute Inc. JMP 10 software.

## 3. Result and discussion

The fatty acid compositions of the studied preserved meat pieces are presented in Table 1.

**Table 1.** Fatty acid composition (g/100 g of total fatty acids) of preserved meat pieces

Fatty acids	Preserved meat pieces (pork) (n = 10)	Preserved meat pieces (poultry) (n = 10)
C14:0	0.94±0.03	0.63±0.28
C15:0	0.05±0.01 <sup>b</sup>	0.09±0.02 <sup>a</sup>
C16:0	23.70±0.50 <sup>a</sup>	22.22±0.41 <sup>b</sup>
C16:1	1.74±0.10 <sup>b</sup>	3.29±0.42 <sup>a</sup>
C17:0	0.31±0.01 <sup>a</sup>	0.13±0.03 <sup>b</sup>
C18:0	13.84±0.92 <sup>a</sup>	7.38±0.93 <sup>b</sup>
C18:1 <sub>n-9</sub>	38.58±1.68 <sup>a</sup>	31.03±1.62 <sup>b</sup>

C18:2 <i>n</i> -6	17.15±0.76 <sup>b</sup>	26.22±0.60 <sup>a</sup>
C20:0	0.31±0.02 <sup>a</sup>	0.10±0.05 <sup>b</sup>
C18:3 <i>n</i> -3 (ALA)	0.99±0.07 <sup>b</sup>	1.80±0.18 <sup>a</sup>
C20:1	0.92±0.04 <sup>a</sup>	0.35±0.03 <sup>b</sup>
C20:2	0.67±0.03	0.52±0.10
C20:3 <i>n</i> -6	0.10±0.02	0.17±0.02
C20:3 <i>n</i> -3	0.14±0.02 <sup>a</sup>	0.07±0.02 <sup>b</sup>
C20:4 <i>n</i> -6	0.41±0.06 <sup>b</sup>	2.22±0.08 <sup>a</sup>
C20:5 <i>n</i> -3 (EPA)	nd	0.19±0.09
C22:5 <i>n</i> -3	0.09±0.01 <sup>b</sup>	0.48±0.03 <sup>a</sup>
C22:6 <i>n</i> -3 (DHA)	0.08±0.07 <sup>b</sup>	0.42±0.03 <sup>a</sup>
SFA	39.14±1.38 <sup>a</sup>	31.56±2.21 <sup>b</sup>
MUFA	41.25±1.74 <sup>a</sup>	37.10±1.61 <sup>b</sup>
PUFA	19.20±0.80 <sup>b</sup>	30.09±0.75 <sup>a</sup>
P/S	0.49±0.02 <sup>b</sup>	0.98±0.06 <sup>a</sup>
<i>n</i> -3	1.30±0.04 <sup>b</sup>	2.35±0.72 <sup>a</sup>
<i>n</i> -6	17.90±0.80 <sup>b</sup>	26.28±2.32 <sup>a</sup>
<i>n</i> -6/ <i>n</i> -3	13.80±0.78 <sup>a</sup>	10.22±1.04 <sup>b</sup>

\*n, number of samples; results are shown as mean±SD; nd = not detected. Values in the same row with the same letter are not significantly different ( $P \geq 0.05$ ). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; P/S = polyunsaturated/saturated fatty acids.

Generally, preserved poultry meat pieces were characterized by lower levels of SFAs. The MUFA content of the poultry meat pieces was significantly ( $P < 0.05$ ) lower than in the preserved meat pieces overall, with oleic acid (C18:1*n*-9) being the most common MUFA. The most common *n*-6 PUFA was linoleic acid (C18:2*n*-6). The most common *n*-3 PUFA was  $\alpha$ -linolenic acid (ALA, C18:3*n*-3), which was more abundant in preserved poultry meat pieces (Table 1), while it occurred in lower amounts in preserved pork meat pieces. Generally, significantly higher contents of docosahexaenoic acid (DHA, C22:6*n*-3) ( $P < 0.05$ ) were found in preserved poultry meat pieces than in preserved meat pieces overall. However, meats and processed meats are also associated with nutrients and nutritional profiles that are often considered negative including high levels of SFAs, cholesterol, sodium and high fat and caloric contents [18]. However, the PUFA/SFA ratios (P/S), as one of the quality parameters of lipid foods, were far greater than 0.4 in the preserved meat pieces (0.49-0.98). The ratio of *n*-6/*n*-3 was 10.22 in preserved poultry meat pieces and 13.80 in preserved pork meat pieces, while the *n*-6/*n*-3 ratio should be between 1 and 4, respectively [19,20].

The estimated percentage of daily saturated fat intake for products is presented in Table 2. Consumption of 100 g of preserved pork meat pieces per day equated to 4.64 g saturated fat that would account for 23.20% of daily fat intake being saturated, while consumption of 100 g of preserved poultry meat pieces equated to 1.73 g of saturated fat, or 8.65% of daily fat intake being saturated, which is closer to the recommended daily intake of 10% of fats being saturated [16,17,21]. Researchers [22] showed that pork products could be modifying to provide a significant increase in functional lipids, which can have positive influences on health.



**Table 2.** Percentage of total fat and saturated fat derived from preserved meat pieces in relation to the reference intake of 2,000 kcal per day, and given 100 g of product is consumed per day

Daily intake	Preserved meat pieces (pork)	Preserved meat pieces (poultry)
Saturated fat, g/100 g of product	4.64	1.73
Saturated fat intake, %	23.20	8.65

#### 4. Conclusion

The increased awareness of the meat industry regarding the importance of the fat quality in processed meat products and its impact on health, optimization of the product specifications (replacement of SFAs with unsaturated fats), health promotion activities by public health authorities, as well as better education of consumers about beneficial nutrition habits (e.g. Mediterranean diet) should reduce the rate of coronary heart disease. Further research is needed to understand better the optimal combination of unsaturated fatty acids in processed meat products and recommended daily intakes that should maintain and enhance the health status of consumers. Studies have indicated that healthy diet has an important role in the prevention of hypertension.

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# The influences of salt replacers on the antioxidative activity of pork

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**Abstract.** Lowering salt intake is of great importance for reducing blood pressure and cardiovascular diseases. The aim of this research was to study the effects of salt replacers on the antioxidative activity of meat. The treatments were formulated with minced pork muscles that were salted with 2.0% salt (control) or different salt mixtures with reduced sodium content (composition 1 – 1.0 % NaCl + 1.2% KCl (experiment 1), composition 2 – 1.0% NaCl + 0.6% KCl + 0.8% CaCl<sub>2</sub> (experiment 2)). After 24 hours of curing, the total antioxidative activity of the antioxidant enzymes was measured. The use of potassium chloride instead of half the sodium chloride did not lead to a significant change in the total antioxidative activity, nor in the activities of glutathione peroxidase and catalase; however, it induced a decrease in the activity of superoxide dismutase ( $p < 0.05$ ). Addition of composition 2 led to a reduction in the total antioxidative activity by 17.7%, and inhibited the activity of superoxide dismutase by 23.8% ( $p < 0.05$ ). The obtained results of the negative effect of the compositions of sodium, potassium and calcium salts suggest the need to develop approaches that allow inhibition of the oxidative changes in meat products with reduced sodium content.

## 1. Introduction

Excessive sodium chloride consumption is associated with an increased risk of hypertension [1,2], which in turn is a crucial risk factor for stroke and other cardiovascular diseases as well as kidney diseases. The necessity to reduce table salt in meat products requires searching for innovative techniques and methods. Among them are a decrease in the amount of salt added in food processing [3,4], a change in the form of the salt, and size of the salt particles [5], the use of taste enhancers [6], and replacement of sodium chloride with mixtures having low sodium content [7, 8].

Taking into account the multifunctionality of table salt in production of meat products, including salt's significant influence on the palatability of the finished product, reduction of sodium chloride will result in deterioration of organoleptic and functional properties of meat products. Thus, many studies assess the expediency of sodium chloride replacement with other chlorides (Ca, K, Mg), which affect taste, water binding capacity, and inhibition of microorganisms. It is known that table salt has many influences on oxidative changes in fat due to salt's inhibitory effect on the activity of the antioxidative enzymes. The antioxidative activity indicates the total protection of the meat system from peroxidation. Reduced antioxidative activity suggests a decreased ability to withstand the oxidative changes. In this context, the aim of this research was to study the effects of salt replacers on the antioxidative activity of meat and on the activity of the antioxidative enzymes (catalase, superoxide dismutase, and glutathione peroxidase).



## 2. Materials and methods

Fresh and post-rigor pork muscles (*Longissimus dorsi*) were taken from female 2-year old Large White pigs in Russia. Meat was ground through a 2-3 mm meat grinder (Vitek, Russia) and salted with 2.0 % edible table for the control group. The other treatments were prepared with the salt compositions as follows: Composition 1 – 1.0% NaCl + 1.2% KCl (experiment 1); composition 2 – 1.0% NaCl + 0.6% KCl + 0.8% CaCl<sub>2</sub> (experiment 2). The prepared minced meat samples were held for 24 hours at 4-6°C.

The extraction was performed according to the method described by Hernandez *et al.* [9] with some modifications. The minced pork samples were subjected to extraction with laboratory dispersing equipment (LDE, Labotex, Russia), using 0.05 M phosphate buffer (pH 7) for 3 min at a 1:5 ratio of volume of extracted sample to extractant solution, at 4-5°C and 5000 rpm. The extract was separated by centrifugation at 15,000 rpm for 15 min at 4.0°C in a centrifuge Sigma 3K30 (Germany). The supernatant was filtered through glass wool Sigma-Aldrich (Germany).

Determination of antioxidative activity was based on the oxidation rate of reduced 2,6-dichlorophenolindophenol (2,6-DCPIP) by oxygen dissolved in the reaction medium. This reaction turns the colorless leuco form of 2,6-DCPIP to the colored form with maximum absorption at 600 nm. The optical density was measured with a photometer (BioChem SA, HTI, USA). Inhibition coefficient (IC) of autoxidation 2,6-DCPIP by the supernatant was the indicator of antioxidative activity.

Determination of the catalase activity was performed according to the method described by Jin *et al.* [10]. Supernatant (0.1 mL) was mixed with 2.9 mL of 11 Mm hydrogen peroxide in phosphate buffer (pH 7.0) at room temperature (22±2°C). The resultant mixture was stirred thoroughly, transferred into the 1 cm cuvettes, and the optical density was measured immediately after the start of the reaction and after 3 min of incubation at a wavelength of 240 nm on a photometer (KFK-3-01 ZOMZ, Zagorsk, Russia). The calculation of the decrease in the hydrogen peroxide concentration took into account the extinction coefficient of 39.4 M<sup>-1</sup>cm<sup>-1</sup>. To measure catalase activity, the amount (mmol) of hydrogen peroxide that was decomposed during 1 min by the supernatant extracted from 1 g of meat was calculated. The results were expressed in U/g of meat.

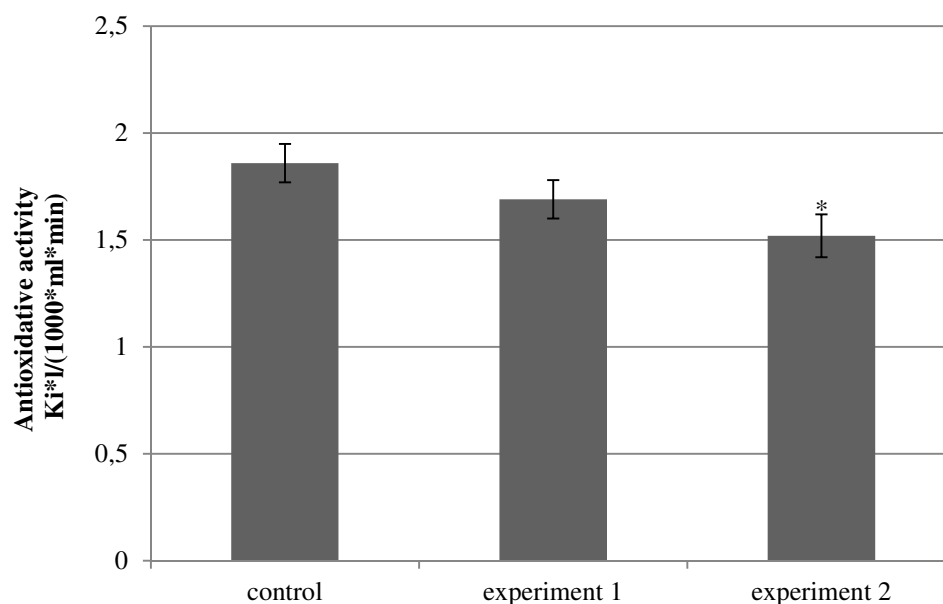
Determination of the superoxide dismutase activity was performed by the method described by Gatellier *et al.* [11], by measuring the pyrogallol autoxidation inhibition. Supernatant (75 µL) was mixed with 75 µL of 10 Mm pyrogallol solution in 2850 µL of 50 Mm phosphate buffer (pH 8.2). The resultant mixture was stirred thoroughly, transferred into 1 cm cuvettes, and the optical density was measured immediately after the start of the reaction and after 2 min of incubation at a wavelength of 340 nm on a photometer (KFK-3-01 ZOMZ, Zagorsk, Russia). Pyrogallol autoxidation was determined in a blank sample of a similar reaction mixture that contained the same volume of distilled water instead of the supernatant. One unit of superoxide dismutase activity is the ability of the sample to inhibit 50% of the reaction. The results were expressed in U/g of meat.

Determination of the glutathione peroxidase activity was performed according to the method described by Jin *et al.* [10]. The reaction mixture contained 1 mL of 75 mM phosphate buffer (pH 7.0), 10 µL of 150 mM reduced glutathione, 10 µL of 46 E/mL glutathione reductase, 30 µL of 25 mM EDTA, 30 µL of 5 mM NADPH, 200 µL of supernatant and 10 µL of 20%-TritonX-100. The volume of the prepared mixture was 1.5 mL. Addition of 50 µL of 7.5 mM H<sub>2</sub>O<sub>2</sub> initiated the reaction. Conversion of NADPH into NADP<sup>+</sup> was registered on the photometer (KFK-3-01 ZOMZ, Zagorsk, Russia) at a wavelength of 340 nm during 3 min, taking into account the extinction coefficient of 6220 M<sup>-1</sup>cm<sup>-1</sup>. One unit of glutathione peroxidase (E) activity is the amount (mol) of NADPH that is decomposed during 1 min by the supernatant extracted from 1 g of meat. The results were expressed in U/g of meat.

All experiments were carried out in triplicate. Statistical processing of the results by determination of the Pearson correlation coefficients and the approximation of reliability was performed using MS Excel for Windows. Assessment of the statistical significance of the differences between parameters was performed using Student's t-test.

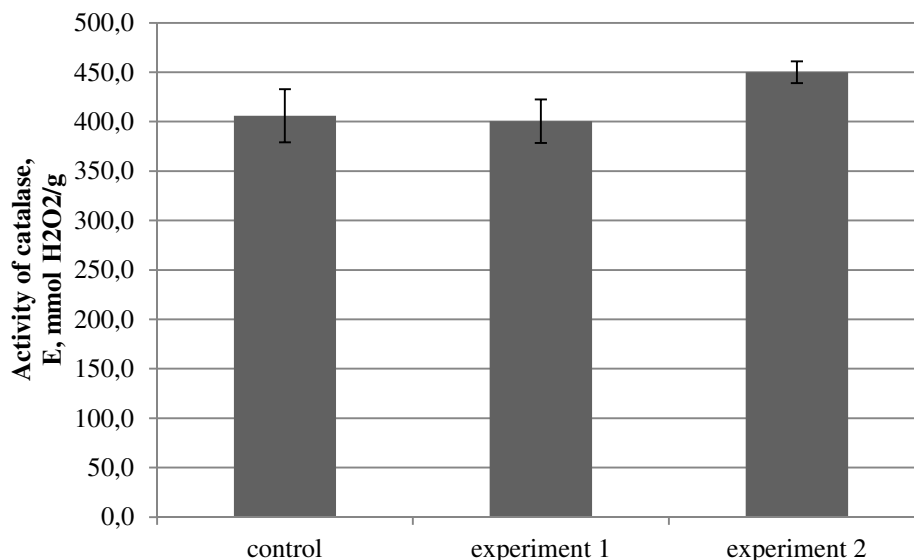
## 3. Results and discussion

The results of determination of the antioxidative activity of the extracts (Figure 1) obtained from the salted meat samples showed that replacement of table salt with potassium chloride did not significantly affect the total antioxidative activity ( $p>0.05$ ). On the contrary, the use of composition 2, which contained sodium, potassium and calcium salts, led to a decrease in the antioxidative activity by 17.7 % ( $p<0.05$ ).



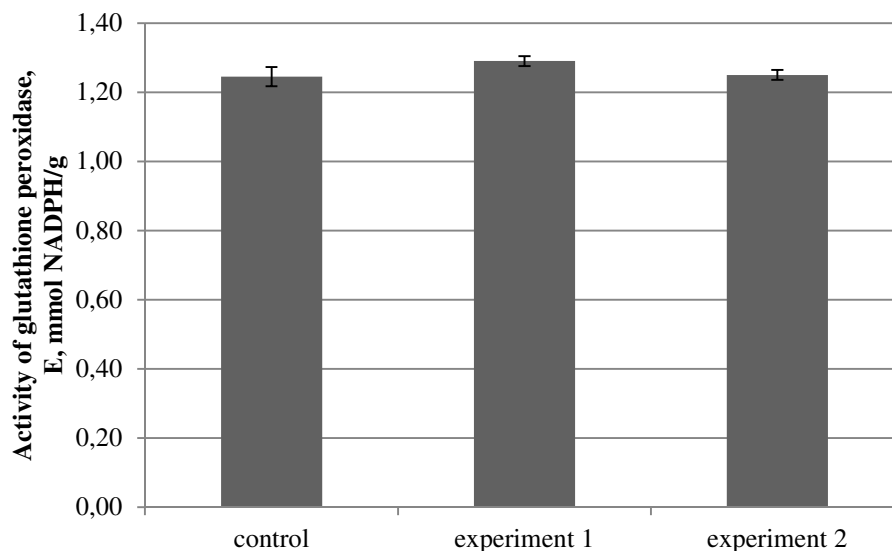
**Figure 1.** The effect of the salt compositions on the antioxidative activity. Experiment 1 – 1.0% NaCl + 1.2% KCl; experiment 2 – 1.0% NaCl + 0.6% KCl + 0.8% CaCl<sub>2</sub> (\*  $p<0.05$  compared to the sample salted with sodium chloride).

Taking into account the significant role of the antioxidant enzymes as a natural form of antioxidative protection, the changes obtained in the meat antioxidative activity in the presence of salt replacers required a more detailed study of the main components of the meat antioxidative system. These are catalase, which catalyzes the breakdown of hydrogen peroxide into water and oxygen; superoxide dismutase, which initiates the conversion of superoxide to oxygen and hydrogen peroxide, and; glutathione peroxidase, which catalyzes the reduction of peroxide by tripeptidylglutathione [12]. The results of the determination of the catalase activity in the salted meat (Figure 3) showed that replacement of table salt with the reduced sodium compositions did not significantly affect the activity of catalase ( $p>0.05$ ).



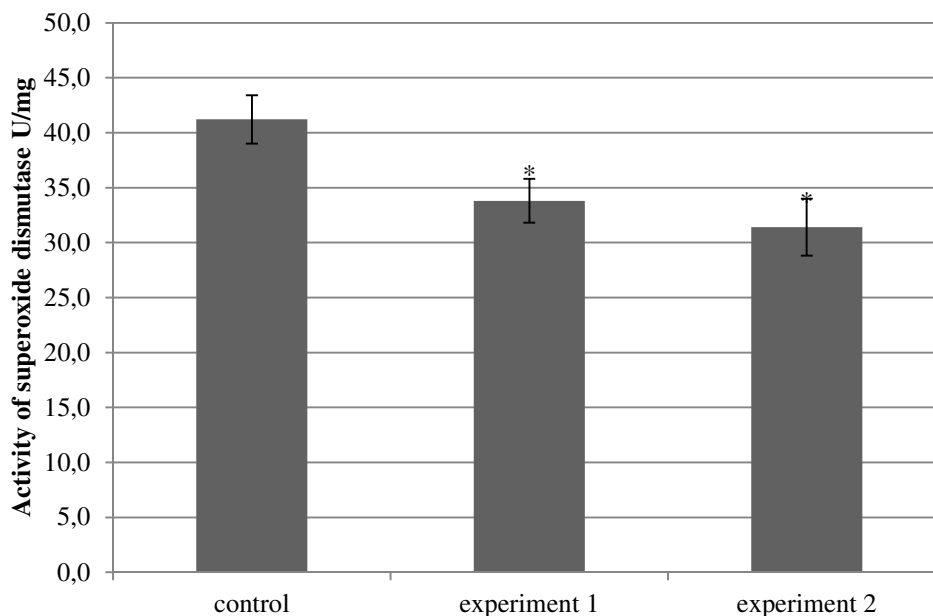
**Figure 2.** The effect of the salt compositions on the catalase activity. Experiment 1 – 1.0% NaCl + 1.2% KCl; experiment 2 – 1.0% NaCl + 0.6% KCl + 0.8% CaCl<sub>2</sub> (\* p<0.05 compared to the sample salted with sodium chloride)

This study also showed replacement of table salt with potassium and calcium salts did not influence the activity of glutathione peroxidase (p>0.05) (Figure 3).



**Figure 3** The effect of the salt compositions on the glutathione peroxidase activity. Experiment 1 – 1.0% NaCl + 1.2% KCl; experiment 2 – 1.0% NaCl + 0.6% KCl + 0.8% CaCl<sub>2</sub> (\* p<0.05 compared to the sample salted with sodium chloride)

However, the table salt replacers did affect superoxide dismutase activity (Figure 4). According to the data obtained, the use of potassium chloride and salts of potassium and calcium for partial replacement of sodium chloride led to a decrease in the activity of superoxide dismutase by 18.0 and 23.8 %, respectively (p<0.05), by the two salt compositions used.



**Figure 4** The effect of the salt compositions on the superoxide dismutase activity. Experiment 1 – 1.0% NaCl + 1.2% KCl; experiment 2 – 1.0% NaCl + 0.6% KCl + 0.8% CaCl<sub>2</sub> (\* p<0.05 compared to the sample salted with sodium chloride)

The increase in the products of oxidation in the meat salted with compositions of salts with potassium chloride and calcium chloride with reduced sodium content could be explained by the decrease in the activity of superoxide dismutase, which was responsible for the reduction in the total antioxidative activity of meat.

#### 4. Conclusion

The results of this study contribute to confirmation of the effects of salt replacers on the oxidative changes in minced pork muscles. Replacement of potassium chloride with sodium chloride did not significantly influence the antioxidative activity of pork; however, it led to inhibition of the activity of superoxide dismutase.

The use of the mixture of potassium chloride and calcium chloride as a salt replacer led to a decrease in the activity of superoxide dismutase and was responsible for a decrease in the total antioxidative activity of meat. Therefore, the use of salt replacers weakened the natural protection of meat from oxidation and, hence, initiated oxidative changes. Since replacement of table salt in meat products will lead to a reduction of the antioxidative protection in the meat raw materials, it will be necessary to use suitable technological approaches that facilitate retardation of oxidation, for example, additional use of antioxidants.

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# The common foodborne viruses: A review

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**Abstract.** Transmission pathways of foodborne viruses include contamination of food by infected food handlers, by contamination of food during the production process and by consumption of products of animal origin harbouring a zoonotic virus. Viral foodborne illnesses, which have become a significant cause of all reported foodborne illnesses in recent years and considered as an emerging risk in veterinary public health. Microbiological genomics studies discovered that Noroviruses and hepatitis A viruses were primarily associated with food-handler transmission and sewage-contaminated foods. In contrast, hepatitis E was associated with consumption of raw or undercooked meat of pig or wild animals. In order to facilitate source attribution and identify risk prevention measures, Routine harmonized surveillance of viral outbreaks, and surveillance of virus occurrence in food commodities, in combination with systematic strain typing, and joint expertise from veterinary, food, and clinical microbiologists would be recommended.

## 1. Introduction

Over the last decades, due to rise of discretionary incomes in Europe and North America, increased urbanization and altered eating habits, worldwide food industry has changed from being locally oriented and supply-driven to become globalized and demand-driven. In order to satisfy consumer groups demanding safety, fair trade, “green” production, and animal welfare-related changes in production practices, policy makers imposed high hygienic standards and various control strategies for pathogenic bacteria, viruses, and parasites. These measures primarily concerned with common causes of food-borne diseases such as unsafe raw food, abused temperature, poor storage infrastructures, inadequate cooking, poor personal hygiene, improper handling methods, and cross-contamination of cooked food with uncooked raw food.

Although contamination prevention and control strategies are mostly successful in reduction of food-borne diseases, they also demonstrate the weakness of the global food supply: if there is a fault in the process, then contamination may occur with pathogens from across the globe, including those that have recently emerged [1]. This proved to be challenging for diverse and complex food systems, especially those in less-developed countries.



In this paper, we will address viral foodborne illnesses, which have become a significant cause of all reported foodborne illnesses in recent years and considered as an emerging risk in veterinary public health.

## **2. Food-borne viruses and their role in food safety**

Currently known viruses that can infect humans are grouped into 22 families. In addition to this, the recent advances in molecular techniques that allow characterization of all genetic material in a given sample has led to the identification of several new viruses in recent years, most of which remain to be fully characterized [2, 3]. Viruses are strict intracellular parasites and cannot replicate outside a specific living cell. The host cell treats viral genetic information as if it were its own. Replication of viruses occurs by transcription and translation of the viral genome using host cell mechanisms. It is not possible to culture them in an environment free of living cells, and therefore the number of viral particles does not increase in food and water during production, processing, transport, and storing. Sensory characteristics of products containing these pathogens and those of non-contaminated food are identical [4, 5]. Transmission of the virus does not only depend on its interaction with the host, but also on the influence of the external environment. Outside the host organism, viruses are inert particles without their own metabolism. The longer they survive in the infectious state environment, the higher is the probability of transmission and spread of infection [6].

Foodborne transmission has been documented for viruses belonging to at least 10 taxonomic families, and the diseases associated with these infections range from mild diarrheal illness to severe encephalitis. Foodborne transmission can occur by:

- Contamination of food by infected food handlers (frequently),
- By contamination of food during the production process (frequently - in bivalve molluscan shellfish or berry fruit production),
- By consumption of products of animal origin harbouring a zoonotic virus (very rare).

The first and second mean of transmission applies to viruses that are transmitted by the faecal-oral route; hence infect their host after ingestion, followed by invasion of cells in the gut epithelium, and subsequent replication in the same site or elsewhere in the body. WHO and FAO [7] have ranked noroviruses (NoV), group A rotaviruses, and hepatitis A viruses (HAV) as priority hazards. When it comes to emerging hazards, hepatitis E virus (HEV), Nipah viruses, H5N1 avian influenza viruses and SARS coronavirus were considered to be of greatest importance. They have also linked risk of specific virus to a specific commodity, and the importance of that commodity in causing viral foodborne illness and found following virus-commodity combinations for which prevention and control measures should be thoroughly considered:

- NoV and HAV in bivalve molluscan shellfish
- NoV and HAV A in fresh produce
- NoV and HAV in prepared foods
- Rotaviruses in water for food preparation
- Emerging viruses in selected commodities

Foodborne NoV outbreaks are often linked to food handlers who infect foods that are eaten raw or not further processed (ready to eat (RTE) foods) prior to consumption [8]. In many of these outbreaks, a sick food-handler or food-handler with a recent history of gastroenteritis was noticed. Workers have often been in contact with ill family members including children before the worker handled food. The most common food worker errors identified in relation to outbreak of NoV and HAV are food handling by an infected person or carrier of virus together with bare-hand contact by handler (RTE foods) and failure to wash hands properly [9]. Poor personal hygiene was also identified as a contributing factor in outbreaks with NoV assigned as the causative agent. Food handlers can

contaminate food either with particles from vomit (NoV) or from faeces (NoV/HAV) when employing insufficient personal hygiene after using toilets. Asymptomatic food workers are implicated more frequently than symptomatic workers, which helps explain the difficulty in detecting and stopping an outbreak by excluding ill food workers [9].

Food contamination at site happens when food is contaminated during the primary production of risky commodities, such as berries, green vegetables or bivalve molluscan shellfish. In these cases sewage or wastewater contamination are the primary source of food-borne viruses, and NoV and HAV were considered as priority concerns according to aforementioned WHO/FAO opinion [7]. Sewage or contaminated water frequently contain multiple RNA viruses, opposite to cases in which food handler contamination occurred. In this case, cohabitation of different (ss+) RNA viruses and subsequent co-infection of human cells by genetically distinct viral strains can lead to the generation of recombinant viruses shuffling their individual mutations and thus making unpredictable effects on viral behaviour and virulence.

Zoonotic food-borne infection occurs when meat, organs, or other products from an infected animal are consumed [10]. For viruses, this is the very rare mode of transmission, although in every emerging disease outbreak this should be investigated. This is especially case with hepatitis E virus since infected pig liver (of both domestic pig and wild boar) consumed raw or undercooked is the main source of infection/contamination. In addition, severe acute respiratory syndrome (SARS) and Nipah virus have been transmitted through food-related incidents [11, 12].

### 3. Norovirus

NoV belong to the family *Caliciviridae*, that is divided into five genera. NoV and Sapovirus are the two genera of the family *Caliciviridae* that contain viruses that cause infections in humans. NoV have also been detected in pigs, cattle, mice, cats, dogs, and sheep, and sapoviruses in pigs. The other genera of the family *Caliciviridae* are Lagovirus, Vesivirus, and Nebovirus encompassing viruses infecting rabbits, and brown hares (lagoviruses), sea lions, swine, cats, dogs, fish, seals, other marine animals, cattle and primates (vesiviruses), and cattle (Nebovirus) [13]. In humans, NoV infection typically causes acute gastroenteritis, with the most common symptoms being nausea, vomiting, diarrhoea, and stomach pain. Symptoms usually develop 12 to 48 hours after infection. The disease normally lasts between 1 and 3 days.

NoV can be divided into five distinct genogroups, based on phylogenetic analyses of the capsid protein (GI-GV). Viruses of GI, GII and GIV are known to infect humans. GII viruses have additionally been detected in pigs, and GIV viruses have been detected in a lion cub and a dog. GIII viruses infect cattle and sheep and GV viruses infect mice. Recombination between viruses from different genogroups is rare suggesting that this constitutes a species level in taxonomy. Within each genogroups, viruses are further divided into genotypes [14].

NoV illness prevalence is highest in young children (< 5 years) and the elderly [15]. Factors that contribute to the significant impact of noroviruses include a large human reservoir, low infection dose (only 10 to 100 viral particles), their environmental robustness, the short-lived immunity to noroviruses (18 months at most), and the ability to be transmitted by various routes. Majority of incriminated foods includes shellfish, which feed by filtration of surrounding water, then berry fruit and green vegetables contaminated during soil fertilizing shortly before picking or watered by contaminated municipal water [5, 16, 17].

Most NoVs can also escape the receptor-blocking activities from antibodies triggered by earlier infections due to accumulated mutations in genome [18, 19]. Viruses are present in faeces and vomitus of diseased people at extremely high levels, up to 10<sup>10</sup> viral particles per gram of stool [20]. The major obstacle to research human noroviruses has been the lack of a robust and reproducible in vitro cultivation system. Such a system is critical to achieve a full mechanistic understanding of human noroviruses replication, stability, evolution and pathogenesis. However, recently stem-cell derived, non-transformed human intestinal enteroid (HIE's) cultures validated as an appropriate pre-clinical model for clinically important enteric infections have been reported [21].

When it comes to prevalence, WHO estimates that Norovirus is the most common cause of foodborne illness in the European region with close to 15 million cases each year, causing more than 400 deaths. In the Netherlands, Norovirus remains the key pathogen causing food-related outbreaks in 2016 as in previous years, followed by *Salmonella* and *Campylobacter* [22].

EFSA, ECDC and FVO have been systematically monitoring whole picture of the state of affairs concerning the Norovirus issue. European Union-coordinated monitoring program on the prevalence of Norovirus in raw oysters was initiated. The objective of the study was to estimate the European prevalence of Norovirus-contaminated oysters at production areas and batches of oysters at dispatch centres, with a 95% level of confidence and a level of precision of 5% considering an expected prevalence of 50%. The survey started in November 2016 and finishes in October 2018 [23]. The EFSA delivered a scientific opinion on the evaluation of heat treatments, different from those currently established in the EU legislation that could be applied to live bivalve molluscs. Of particular relevance are the achievement of at least 90°C for at least 90 s in the molluscs flesh and the inactivation of viruses [24].

Currently, EU regulatory authorities are focusing in following areas in Norovirus combat: (i) whole genome sequencing for the characterization of Norovirus and other foodborne viruses; (ii) surveillance to generate more information about levels of Norovirus occurring in food; (iii) refinements to current RT-PCR to improve detection of low numbers of norovirus particles in all food matrices; (iv) the binding properties and possible methods of inactivation of norovirus; (v) the effectiveness of depuration (or alternatives such as high pressure, UV, ozone, irradiation) in removing Norovirus from oysters; and (vi) establishment of the infectious dose in different food commodities including shellfish and fresh produce (lettuce and berries).

#### 4. Hepatitis A virus

The hepatitis A virus (HAV) which belongs to genus *Hepatovirus* within family *Picornaviridae* causes hepatitis A. *Hepatovirus* have only been found in humans and primates, suggesting there is no introduction from any other reservoir. Based on genetic diversity, hepatitis A viruses are divided into six lineages or genotypes; of which genotypes I-III infect humans [25]. It consists of a non-enveloped icosahedral capsid of around 30 nm in diameter containing a positive ssRNA genomic molecule of 7.5 Kb [26]. HAV is a unique picornavirus because it does not inhibit host-cell protein synthesis to allow a regulated ribosome traffic rate thus ensuring the proper protein folding [27]. Capsid folding is critical to permit a long period-environmental stability for a virus transmitted through the faecal-oral.

Since proper sanitation and good hygienic conditions greatly reduce transmission rate of HAV its prevalence is significantly lower than prevalence of NoVs [28]. In highly endemic regions, HAV is one of the childhood infections that, in the majority of cases, runs an asymptomatic course, while triggering a protective immune response that lasts long, possibly even lifelong [29]. HAV is quite stable outside a host and, therefore, can persist on contaminated environments, food, and water for a quite long time. Food- and water-borne outbreaks have been documented, although again, as for NoVs, the most common mode of transmission occurs between persons. Incidence risk of food-borne HAV at present comes from introduction through food into regions where population immunity is relatively limited. Foods commodities susceptible to contamination during the production phase, such as bivalve filter-feeding oysters, clams, mussels or commodities that are irrigated with water that may be contaminated (lettuce, green onions, and soft fruits, such as raspberries and strawberries). These foods should be considered the principal targets for virological analysis. Nevertheless, in roughly 40% of the reported cases of hepatitis A the source of infection cannot be identified [30]. The first documented shellfish-borne outbreak of “infectious hepatitis” occurred in Sweden in 1955, when 629 cases were associated with raw oyster consumption. However, the most significant outbreak of HAV infection occurred in Shanghai, China, in 1988, in which almost 300,000 cases were caused by consumption of clams harvested from a sewage-polluted area. A specific problem with shellfish is that the current microbiological quality control criteria are based on quantitative testing for *E. coli*

contamination, which often fails to predict the presence or absence of viruses. Water-depurated shellfish have been associated with outbreaks of Norovirus, hepatitis A, gastroenteritis, and other viral diseases [31].

## 5. Hepatitis E virus

HEV is a non-enveloped icosahedral virus with a diameter of 35 nm, classified into the unassigned genus *Hepevirus*. The genome consists of one positively oriented single-stranded RNA molecule and around 7 kb in length. The major ORFs are ORF-1, which encodes a non-structural polyprotein, ORF-2 encoding the capsid protein and ORF-3 encoding a phosphoprotein.

The HEV strains can be grouped into 4 genotypes, with different spatiotemporal distribution and different host. Genotypes 1 and 2 have been found solely in humans, i.e. genotype 1 is endemic in Asia and Africa where inhabitants are exposed to the virus due to poor sanitary conditions and sewage overspill that results from heavy rainfall [32]. In these conditions, surface water is contaminated that is used for drinking water production or as source for water used for household tasks, so this explains the magnitude of outbreak. Genotype 2 is endemic in Mexico and Western Africa. However, beside in humans, genotypes 3 and 4 have been detected in pigs and other animal species. Genotype 3 is distributed worldwide and genotype 4 is mostly restricted to Southeast Asia. Endemic strains found in Europe are usually of genotype 3.

The epidemiology of HEV is rather complex, and a foodborne transmission of HEV from animal products to humans is an emerging risk, especially in the European developed countries. A few research studies indicated the following food commodities present risk factors for onset of HEV infection: pork pies, liver pate, wild boar, under-cooked or raw pork, home-made sausages, meat (in general), unpasteurized milk, shellfish and ethnic foods [33]. Nevertheless, these factors were not adequately proven due to scarce data obtained from very few systematic studies. One systematic case-control study has been performed in Germany, in which eating of any offal or wild boar meat was identified as risk factor for autochthonous hepatitis E [34]. In addition, another recent small-scaled case-control study identified eating of raw pig liver sausage as a risk factor for hepatitis E in France [35]. Earlier publications from Japan indicate direct HEV transmission by eating raw or undercooked meat from wild boar or deer by detailed analysis of small outbreaks [36]. No detailed information on hepatitis E cases, including the proportion of foodborne cases, is available for the EU, which is the reason why EFSA in July 2017 advised national competent authorities to commence gathering data on HEV prevalence and/or possible HEV outbreaks [37]. Despite rough estimations that approximately 2 billion people could have been exposed to HEV [38], majority of HEV cases occurred in the endemic regions in Asia, Africa and Central America, where transmission is mainly due to faecal-contaminated water. Europe is not a endemic region, but sporadic hepatitis E cases have been described in France, The Netherlands, Spain, Hungary, the UK, Denmark, Norway [39], indicating an EU-wide distribution of the virus. In Germany, HEV is notifiable as of 2001 and their data indicate that 40 to 220 cases (mostly non-travellers in endemic area) per year are registered, with increasing tendency [39]. In France, the disease is also notifiable and 218 cases have been identified in 2008. Among these cases, 146 have been identified as autochthonous cases, 23 to travels and no epidemiological data was available for 49 cases [39].

## 6. Conclusion

NoV and HAV have been recognized as priority concerns in viral food-borne transmission. However, proper diagnosis of infection caused by these agents is often hindered due to sharing general symptoms with other diseases (fatigue, dehydration, nausea, vomiting, diarrhoea, and some stomach cramping), failure of notification and relatively quick resolution of signs of illness. The most important role in transmission route is attributed to infected food handlers and sewage-contaminated foods. In the latter category, complex mixtures of human and animal viruses and other pathogens may be present in a single food item, causing possible genetic recombination and subsequent uncontrolled expansion of the diversity of these pathogens. Routine harmonized surveillance of viral outbreaks, and

surveillance of virus occurrence in food commodities, in combination with systematic strain typing, and joint expertise from veterinary, food, and clinical microbiologists are highly recommended to aid source attribution studies and identify risk prevention measures.

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## Air quality and impact on food safety

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**Abstract.** Air is an important potential source of contamination in different areas of human endeavor (medicine, pharmaceuticals, agriculture, food industry etc.). During the various technological procedures of producing and processing food, its contamination by polluting substances contained in air, such as suspended particles (physical contamination), combustion products and volatile organic substances (chemical contamination) and biological pollutants (bacteria, viruses, molds and mites) very often occurs. Although the sources of air contamination are numerous (poor construction aspects of the building, improper air conditioning and ventilation, etc.), basic and common sources are people and their activities in food production plants. The task of responsible food production is to adequately and continuously implement good production practices, and thereby, prevent potentially harmful practices, to ensure environmental health and safety for the life and work of the staff in that environment, and to create conditions for safe food production, while protecting the environment.

### 1. The most common air pollutants

Cleaning and sanitation of that food contact surfaces is an integral part of mandatory sanitary procedure in the food industry. However, is the same level of concern aimed at preventing the occurrence of air contamination or at air contamination control during the food production process? The real answer is no, or not enough.

According to the World Health Organization [1], air-polluting substances are considered to be materials, the presence of which in specific concentrations can have direct or indirect negative consequences for human health and the environment. The data suggest that polluted air, on an annual basis, is responsible for 7 million premature deaths around the world, placing it as one of the eight most important etiological death factors.

Primary air pollutants in the food industry are marked, being, in addition to microorganisms, suspended particles, combustion products (nitrogen oxide, carbon monoxide, carbon dioxide, sulfur oxide) and volatile organic substances. Bacteria can be found individually in the air, but also occur as agglomerates on solid (e.g. dust) or liquid (e.g. condensed water) microscopic particles scattered in the air [2]. In addition to bacteria occurring in specific *bioaerosols* (synonym, organic dust), these particles can contain bacterial spores, fungi and/or their spores or hyphae, viruses, different types of antigens, pollen grains, toxins, fecal material or their combinations [3, 4]. Metabolites and secretions are also included in this context [5]. The size of aerosol particles is generally in the range 0.5-50 µm



[6]. It was long thought the size of particles of microorganisms in the air was the same as the microorganisms themselves. However, very often this is not the case, so the sizes of particles containing microorganisms in the air are frequently much larger than the microorganisms. For example, a bacterial cell with a diameter of  $\sim 1 \mu\text{m}$ , on being bioaerosolized in water, produces a water droplet with diameter of 15-25  $\mu\text{m}$ . Skin particles, according to Noble [7], have a mean equivalent diameter of 13.5  $\mu\text{m}$  and fewer than 30% of the particles carrying staphylococci were less than 10  $\mu\text{m}$  in equivalent diameter.

Different aspects of the technological process of food production, starting from manipulation of raw materials, processing stages (measuring, grinding, mixing, etc.), storage, transport, and sales of finished product to the consumer are risk phases in which the air is a significant source of microbiological contamination. That is what, more than one and a half centuries ago (1861), in *Annales des Sciences Naturelles*, Louis Pasteur [8] wrote about when presenting his first research on the presence of microorganisms in air.

The most common means of spreading microorganisms in food production plants is through various construction openings (doors, drains, etc.), disinfectant tunnels, during cleaning processes, washing and packaging, due to poorly designed and poorly maintained ventilation and air conditioning systems, and poorly constructed interior and roof structures (drainage, leakage) etc. [9]. However, the basic and most dangerous sources are people and their activities in food production plants [10]. The microorganisms are, in this case, suspended on particles of dust, hair, shoes or clothes originating from the workers, as well as in the droplets that are formed during talking, coughing or sneezing [11]. Also, all manipulative actions that aerosolize contaminants lead to unacceptable levels of microbial pollution in food production plants [12], so it is desirable to maintain dry conditions.

In Table 1, an overview of the most common sources of microbiological contamination of air in the food industry, the principles of their transfer, and their consequential level of risk on product is given.

**Table 1.** Influence of sources of contamination and air ventilation system producing risk to product safety [12]

Source of contamination		Transmission mechanism	Ventilation system effect	Risk for product safety	
Material etc.)	(packaging,	Material surface	Low	Medium to high	
Workforce		Clothes, footwear, and enhanced due to poor personal hygiene	Low	Medium to high	
Internal transport		Manipulation by transport assets	Low	Medium to high	
Air inside facilities		Fresh air	High	Medium	
		Dust or powder	High	Medium	
		Spray aerosolation	Medium	High	
		Pneumatic transport, crimping	High	High	
Condensation		Contact	High	High	
Equipment material	surface	Contact with materials	Low	High	
Cleaning operations		Spray-splash, washing, vacuolation	High	High	
Equipment machines	and	Pneumatic gas exhaust systems, compressed air	High	Medium to high	
Building appearance		Building construction, poor ventilation via windows and	Low	Medium to high	

doors, poor building design and construction

Temperature, relative humidity, sources of nutrients, and air movement affect the growth and dissemination of airborne contaminants. In other words, unless favorable nutritional conditions are present, the air is not a natural environment for growth and reproduction of microorganisms [2]. On the other hand, if there are adequate conditions for survival and multiplication of microorganisms, there is a potential risk that contaminated air in food production environments will lead to public health burdens on the staff, but also to the possibilities of food contamination or reduced quality and/or shelf-life of the food product [13, 14].

## 2. Most common microorganisms involved in air contamination in the food industry

Although pathogenic and spoilage microorganisms can come in contact with a finished food product in different ways [15], the air is often cited as a significant source of microbiological contamination, especially in plants processing milk and dairy products [16], pork [17], poultry [18], and beef meat [19]. The microbiological air quality, from the aspect of acceptable level of contamination, in food industry facilities, is proportional to the level of product risk. Therefore, for a low-risk food, such as packaged, canned products, low-value products that are stable at room temperature, or products that are required to undergo thermal processing by consumers, the air system can be permitted less challenging performance requirements. On the other hand, when producing high-risk foods, such as ready-to-eat foods, the air quality level must be high. Thus, factories producing medium or high risk foods very often utilize physical separation of the production units [20].

The interconnection between microbiological contamination of food products and the spread of microorganisms in the air environment is presented in Table 2.

**Table 2.** Microbiological contamination of air that comes into contact with food products [21]

Air contamination	Processes/Rooms	Total microorganisms, CFU <sup>a</sup> /m <sup>3</sup>	Yeasts and molds, CFU/m <sup>3</sup>	Relative humidity, %
Low	Packaging milk products	100-40	10-40	50-70
	Preserving meat products	150-300	30-120	45-60
	/laboratory control			
	Fresh meat delivery	300-600	100-500	45-60
Medium	Meat processing	800-1800	250-500	70-80
	Meat shops	400-900	150-500	50-60
	Gastronomic food	500-1100	200-600	55-65
	Bars	600-1000	150-450	50-60
	Slaughterhouses	1500-6500	600-1900	55-65
High	Sausage production	1500-3500	2000-10000	70-80
	Internal organ processing	4000-6000	700-3500	55-70
	Market	1500-7000	500-5000	50-70

<sup>a</sup> CFU – colony-forming unit

The contamination of food products is dependent on air's microbial load, and on the duration of exposure to the air, whether during specific technological processing stages (e.g. cooling) or during storage [22]. For example, the rate of food contamination is increased by 120% after 48 h of air exposure at 32°C, compared to the same conditions with 24 h exposure [2].

Microbiological contamination is higher given greater air humidity, in which conditions, 80-95% of isolated microorganisms are Gram-positive bacteria. The air in slaughterhouses is contaminated mostly with Gram-negative airborne bacteria belonging to the families *Enterobacteriaceae* and

*Pseudomonadaceae*, while the Gram-positive airborne bacteria present belong to the genera *Staphylococcus*, *Microbacterium*, *Bacillus* and *Micrococcus* [23]. The most significant species are *Bacillus megaterium*, *Bacillus brevis*, *Micrococcus luteus* and *Micrococcus varians*.

Gram-negative bacteria that produce endotoxins were determined in the air of poultry slaughterhouses, and they are the consequence of air being contaminated with feces from slaughtered poultry [24, 25]. To reinforce this point, high numbers of bacteria, especially *Staphylococcus* and *Corynebacteria*, were also determined in the air of broiler farms [26].

A large number of molds (11 orders and 32 species) were determined in cheese ripening facilities [27]. Most species (45.16%) belonged to the genera *Penicillium* (the most dominant species was *Penicillium verrucosum* var. *cyclopium*) and *Aspergillus*. Air quality is particularly important in facilities for production and packaging of butter, if this is manufactured in open-type mixers, since these devices can also incorporate up to 5% of the surrounding air into the product [28]. In terms of storage of cooled butter products, i.e., in refrigerators or other cooling chambers, the predominant molds belonged to the genera *Penicillium*, *Aspergillus*, *Mucor* and *Cladosporium* [29, 30].

### 3. Conclusions

This summary of existing information on the bioaerosol contamination in food processing plants concludes that existing data in Serbia, however, are limited, mainly because of the lack of appropriate equipment for sampling, and lack of expertise in procedures for bioaerosol examination, but partly because of fear that the outcomes of such studies would negatively affect food producers. On the other hand, consumer demand for microbiologically healthy food is increasing, and this is also an expressed imperative of food producers. Therefore, regardless of whether bioaerosols constitute a small or high risk for food contamination, it is necessary to implement permanent air control while taking appropriate measures to comply with minimal quality standards, and thereby, limit and/or prevent the possibility of microbiological contamination of food.

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# Current information levels on honey labels in Vojvodina

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**Abstract.** Honey sells according to its appearance and the information given on the label. Labels provide the necessary information to consumers to enable them to make safe, healthy and sustainable choices. Label information is usually the only information consumers have at their disposal when deciding whether to buy honey. Lack of any information on honey labels causes confusion and misunderstanding among consumers. A total of 103 labelled honeys were collected from different retail chains and market places in Vojvodina, Serbia, in autumn 2018. All honey labels were analysed according to the requirements of Serbian food labelling regulations. Generally, the labels did not fulfil legal labeling requirements. Specifically, 89.32 % of labels lacked at least one of the mandatory pieces of information from the list stated in the regulations. This result shows the need for further education of beekeepers, manufacturers and distributors of honey.

## 1. Introduction

Honey is natural sweet substance made by bees on collecting nectar of plants, combining it with their own substances and depositing, dehydrating and storing it in honeycombs to ripen and mature [1].

In Serbia, 7014 tons of honey were produced in 2017, i.e. 8 kg of honey was produced per beehive. Compared to 2016, when 7 kg of honey was produced per beehive [2], it could be that honey production is slightly increasing. The market for locally produced honey is important in Serbia. Local beekeepers sell their own honey *via* retail chains, farmers' markets, roadside stands or their homes. In the province of Vojvodina (north Serbia), in 2017, the sale and purchase of honey and beeswax amounted to 240 million RSD [2].

Honey is used as a food and natural health product, due to its high-calorie, easily digestible properties and composition [3]. Consequently, besides safety and quality control of honey, adequate and correct information present on honey jar labels is equally important.

Information on labels can provide a wide range of information and help consumers avoid suspect honey, such as adulterated and misbranded honey [4]. The total amount of information that has to be included on honey labels has become complex and comprehensive. Details are prescribed by law, but problems with labelling still occur in Serbia. The current legislation in Serbia sets out the requirements for the labelling, advertising and presentation of foodstuffs, including honey and honey products. The food business operator, or the operator under whose name or business name the food is marketed, is responsible for food information in accordance with the applicable food information law and requirements of relevant national provisions [5, 6].

Information on labels can be divided into mandatory and voluntary. Mandatory information must always be displayed on labels, while voluntary information may or may not be displayed. Food labelling policies have a dual purpose: to protect consumers and to ensure fair marketing. Some of the



information, such as batch number, establishes the traceability of honey. Traceability is the ability to trace every step forward through the distribution chain, from origin to destination, and backward, providing information on origin of the materials, ingredients and processing history of the products [7].

Considering honey sells according to its appearance and the information given on the label, labels can help each honey product to stand out from the competition. Therefore, attractive, informative and effective labelling is important [8].

In March 2017, the Serbian government adopted the rulebook on food declarations, labelling, and advertising [5], and planned it to be fully implemented on June 15, 2018. A year later, a change to this rulebook was adopted. Therefore, according to the latest legislation, all the packed and labelled food, until the date of the application of the rulebook from 2017, which does not meet the rulebook's conditions, can be released on the market until its expiration date, but no later than December 31, 2018.

Regarding this deadline, the aim of this study was to investigate current information levels on honey labels in Vojvodina, Serbia.

## 2. Materials and methods

In total, 103 labelled honeys (Table 1) were collected from different retail chains and market places in autumn 2018 from Vojvodina (north Serbia).

**Table 1.** Types and numbers of honey with labels that were analysed

Type	Number
Meadow honey	35
Acacia honey	34
Linden honey	14
Honeydew honey	6
Honey with no statement of identity	4
Floral honey	3
Sunflower honey	3
Baker's honey	2
Rapeseed honey	1
Linden-meadow honey	1
Total	103

Every honey label was inspected for the presence of statements of identity, classification according to origin and according to mode of production and market placement, net weight, shelf life date indication, storage condition, batch number, manufacturer's or distributor's name and address, and country of origin.

## 3. Results

The results obtained (Table 2) showed the low compliance of the honey labels with regard to the mandatory statements that must appear on honey labels under current regulations [5, 6].

**Table 2.** List of the mandatory information elements contained on the honey labels

Information	Number of labels without information	% of labels without information
Name	4	3.88
Classification according to origin	78	75.73
Classification according to mode of production and market placement	57	55.34
Net weight	25	24.27
Shelf life date	42	40.78
Storage conditions	75	72.82
Batch number	75	72.82
Manufacturer's or distributor's name and address	2	1.94
Country of origin	66	64.08

Out of 103 examined honey labels, 92 (89.3 %) lacked at least one of the mandatory pieces of information from the list prescribed in the regulations [5, 6].

The manufacturer's or distributor's name and address and the name of honey were the items most commonly present on the labels, followed by net weight information and shelf life date. However, more than half of the honey labels (55.3 %) did not contain information about classification according to mode of production and market placement.

Most of the labels did not have any information about the classification of honey according to origin (75.7 %), storage condition (72.8 %) and batch number (72.8 %).

The results show that only 11/103 (10.7 %) honey labels fulfilled the legal labelling criteria in Serbia [5, 6].

#### 4. Discussion

Despite more than one year of preparation, many honey labels in this study lack even basic information required by the legislation. A study conducted in 2017 [8] showed better results, but still low compliance with the law, with 23.33 % of labels in accordance with legislation.

Mandatory label information allows consumers to make healthy and informed dietary decisions [9]. The first five pieces of food label information that consumers look at are expiration date, production date, shelf life, name and brand of product and ingredients [10]. This set of information conveys to consumers the product's characteristics and influences consumers' purchase behavior [11].

In this study, four honey labels did not contain name information. Products conforming to the definition of honey must be designated "honey". The name "honey" can be supplemented by the term "blossom" or "nectar" [6]. The floral or plant name can be included in the honey name if the honey comes wholly or mainly from that floral or plant source, and has the organoleptic and physicochemical properties corresponding with that origin [1].

The extraction of honey includes separation of the honey from the combs by pressing. Honey extracted from combs contains pollens, beeswax, and other undesirable materials. To ensure better quality and longer shelf life, these undesirable materials should be removed [12]. The most common method of processing honey is by centrifuging decapped broodless combs. All the honeys from this study that included information about processing on their labels were extracted honeys.



Due to honey's nutritional value, health benefits, the growing global trade and the economic gains, adulteration of honey has increased. Adulteration of honey is relatively easy to conduct, but difficult to detect. The most frequent frauds are based on preparations with simple and complex sugars, which can mimic the natural sucrose-glucose-fructose profile of honey [13, 14, 15].

Thus, honey authenticity is a globally important concern for consumers and honey producers. Honey authenticity has two aspects: origin of the honey and the mode of production of the honey. The origin of the honey covers geographical origin and botanical origin, while production mode is related to the harvesting of honey hives and processing [6]. Specific flora and vegetation in the area from which the honey originates determines the quality of honey, and affects its organoleptic properties [14]. Although, the presence of this information regarding honey's authenticity on labels cannot guarantee the quality, it can gain consumers' trust. In Poland, the geographical origin was seen as the most useful and interesting information on honey labels [15].

Properly processed, packaged and stored honey retains its quality for a long time. Honey should be stored in airtight containers. This protects it from external moisture that the honey can absorb, and odors. Optimum storage temperature is 10-16 °C, while the relative humidity of the storage room should be <65 %. However, honey is susceptible to physical and chemical changes during storage. This is a temperature-dependent process, making the shelf life of honey difficult to define. With increasing temperature, the 5-hydroxymethylfurfural content increases, while the enzyme activity decreases. This decreases the quality of honey [16]. Honey should retain its specific properties for a number of years if correctly stored. It is up to the beekeeper or honey packer to determine a suitable shelf life for their product. In our study, honey labels that did contain information about storage conditions cited room temperature storage as being optimum.

Net weights of 78/103 (75.7 %) of our honeys were expressed in gram (g) or kilogram (kg). Better results were obtained in an earlier study [8], where 96.67 % of inspected labels contained net weight information.

Traceability is important for safety, quality and labelling. The ability to physically trace honey in every step from origin to destination is important to consumers. Although the information remains in the ownership of the beekeeper, manufacturer or distributor, consumers are willing to pay extra for honey with a traceability system [17].

Food labelling frameworks aim to regulate different interests, which range from human health and consumers' rights to international trade. However, consumers admitted that they purchase honey in containers without labels, but indicate that the honey came from a trusted manufacturer or retailer [18, 19].

## 5. Conclusion

The purpose of legal labelling requirements is to provide consumers with important information about the honey they purchase. This study shows that some honey labels do not fulfill regulatory requirements and, at the same time, we feel they would be unlikely to gain consumer trust. Honey labelling is costly, and further expenses have to be met when the labelling is not appropriate, especially in the cases of formal charges for a labelling offence or if product withdrawal from the market is required. This research provides a foundation for further education of beekeepers, manufacturers and distributors of honey.

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## Evaluation of Serbian black locust honey quality parameters as a contribution to confirmation of its botanical origin

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**Abstract.** Acacia honey, like many other specialized kinds of honey, is derived purely from one plant species, in this case, the nectar of black locust (*Robinia pseudoacacia*) tree flowers. The present study investigated the quality of 270 acacia honeys from the Serbian market, collected during 2017 and 2018. Chemical and physical properties of honey were evaluated according to Serbian regulation. All applied methods were performed according to the Harmonized Methods of the International Honey Commission. Summarizing the results presented, none of the tested acacia honeys exceeded limits of national or EU regulations for moisture, free acid and insoluble matter contents as well as electrical conductivity. However, the most common parameters for non-compliant honeys were hydroxymethylfurfural and sugar contents and diastase activity. Among these parameters, this study shows the fructose to glucose ratio is also an important quality factor, significant for confirming the origin of acacia honey, while the correlation between glycemic index and the fructose to glucose ratio is especially important for individual honey consumers with impaired glucose tolerance or insulin resistance.

### 1. Introduction

Honey is defined as “the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plantsucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honey combs to ripen and mature” [1]. The major components of honey are sugars, mostly fructose and glucose, although other minor components such as enzymes, proteins, organic acids, minerals, pollen grains, waxes and phytochemicals are also present [2]. The composition of honey is characterized by the type of plants from which bees collect their nectar, climatic conditions, environmental factors, and bee farming practices [3, 4]. Acacia honey, like many other specialized kinds of honey, is derived purely from one plant, in this case, nectar from flowers of the black locust (*Robinia pseudoacacia*) tree, which is native to North America, but is also present in Europe, where it is known as a “false acacia”. For clarity reasons, honey originating from *Robinia* will be, hereinafter, referred to as acacia honey. This variety of honey is almost clear, like liquid glass, and has a mildly sweet flavor.

The Community Directive 2001/110 [1] and national regulation [5] strictly defined and established the general and specific characteristics of honey and the quality indicators which characterize individual honey varieties. Regulative recommendations are that acacia honey should have a moisture content not more than 20%, sugar content not less than 60%, sucrose not more than 10%, free acidity



not more than 50 meq/kg, hydroxymethylfurfural (HMF) content not more than 40 mg/kg, water-insoluble content not more than 0.1%, diastase activity not less than 8 Göthe units and electrical conductivity not more than 0.8 mS/cm.

Limited acacia honey production due to its rarity and insufficient availability has produced heightened interest in its adulteration [6]. Honey adulterants are mainly starch syrup, invert syrup, starch or invert syrup fed to bees, and low quality honey added to high priced honey [7]. The present study shows the results of an investigation into the quality of acacia honey from the Serbian market in 2017 and 2018. Chemical and physical properties of honey were evaluated according to Serbian Regulation [5] and in order to select parameters important for confirming and distinguishing acacia honey from other blossom honeys characteristic of Serbia and the Balkan region.

## 2. Materials and Methods

### 2.1. Honey samples

A total of 270 acacia honey samples were obtained from different regions from the Serbian retail market during 2017-2018. All honeys were stored at ambient temperature prior to analysis. In most of the honeys, all parameters of quality defined by the legislation were examined, and in a smaller number, analyses were carried out as per client's request.

### 2.2. Methods of physical and chemical analysis

All applied methods were performed according to the Harmonized methods of the International Honey Commission [8]. The moisture content (%) was determined from the refractive index of the honey by reference to a standard table. Free acidity was determined by titration to pH 8.30 and expressed as milli equivalents/kg (meq/kg). Electrical conductivity (mS/cm) was performed using a conductivity meter at 20°C in a 20% (dry matter basis) solution of honey samples prepared with ultrapure water. Content of insoluble matter (%) is defined as that material found by the procedure to be insoluble in water. Determinations of sugars (glucose, fructose and sucrose) (%) were performed with a Waters 2690 high-performance liquid chromatograph equipped with a refractive index (RI) detector (Waters model 2414). Duplicate injections were performed and average peak areas were used for the peak quantification. Glucose, fructose and sucrose, purity  $\geq 99.5$  % (Sigma–Aldrich), were used as standards to determine the sugar content of honey. Quantification was performed according to the external standard method on peak areas. Determination of diastase activity after Schade was performed and results are expressed in Göthe units per gram of honey (DN). The concentration of 5-(hydroxymethyl)-furan-2-carbaldehyde (HMF) was determined using reverse phase HPLC equipped with UV detection and the result is expressed in mg/kg. Carbohydrate ratios (F/G) were calculated and evaluated with respect to literature data. All the tests were performed in duplicate and expressed as minimum, maximum, mean and median values.

### 2.3. Statistical analysis

For statistical evaluation of data, Microsoft Excel with Data Analysis Tool Pack from MS Office was used.

## 3. Results and Discussion

The results of physicochemical analysis expressed as minimum, maximum, mean and median as well as number of tested and compliant honeys are presented in Table 1. All studied acacia honeys were within standard limits of moisture, free acidity, electrical conductivity and water insoluble content [5]. None of the honeys exceeded the maximum allowed moisture value of 20% [1, 5], levels above which can elevate the honey's ability to resist fermentation and granulation and impede longer shelf life during storage [8]. These values agree with the results obtained in 201 acacia honeys from Serbia, tested in 2009 (average moisture content 16.12%) [9] and 132 acacia honeys in 2014-2016 (moisture content in all examined honeys were lower than the permitted value) [10]. Slightly higher mean values

for moisture content ( $16.00 \pm 1.33\%$ ) were reported in examined acacia honeys from Bosnia and Herzegovina [11], Spain ( $17.5\%$ ) [12], and Romania ( $17.9\%$ ) [13].

**Table 1.** Descriptive statistics for acacia (*Robinia pseudoacacia*) honey quality parameters and number and percent of compliant honeys

Parameter	Number of samples examined	Min	Max	Mean	Median	Compliant (number)	Compliant (%)
Moisture (%)	196	13.30	20.00	16.31	16.22	196	100
Free acidity (meq/kg)	180	3.00	45.90	10.78	9.89	180	100
Electrical conductivity (mS/cm)	222	0	0.66	0.17	0.16	222	100
Water-insoluble content (%)	191	0	0.06	0.01	0.01	191	100
Glucose and fructose content (%)	196	32.62	80.55	69.59	70.23	190	96.94
Sucrose (%)	200	0.97	42.12	6.37	5.95	196	98.00
Fructose/ glucose ratio	196	0.44	1.91	1.52	1.60	149*	76.02
Diastase activity (DN)	197	0	50.16	15.70	13.98	191	96.95
HMF (mg/kg)	210	0.16	295.2	13.18	4.49	197	93.81

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\* Honeys with fructose to glucose ratio over 1.4 were considered as compliant [14]

Free acidity is a quality parameter related to honey fermentation [15]. However, the presence of different organic acids, geographical origin and harvest season can affect the honey's acidity [16]. National regulation permits a maximum value of 50 meq/kg [5]. The results obtained (mean value was 10.78 meq/kg) showed a similar trend to data from a study of quality in honey from the Serbian retail market, 2014-2016 [10], when reported mean values for free acidity for acacia honeys were 10.82 meq/kg (2014); 10.87 meq/kg (2015) and 8.23 meq/kg (2016). A slightly higher mean free acidity ( $12.43 \pm 7.13$  meq/kg) was shown for acacia honey from Bosnia and Herzegovina [11], while free acidity was lower (6.45 meq/kg) in acacia honeys from Romania [13].

Electrical conductivity is often used in the quality control of honey to distinguish blossom honey from honeydew [17] and is considered a good criterion for the botanical origin of honey [18]. The Official Regulation on quality of honey [5] recommends a maximum value of 0.8 mS/cm for acacia and blossom honey and minimum value of 0.8 mS/cm for honeydew. The mean electrical conductivity value of 0.17 mS/cm was similar to our previously reported electrical conductivity levels of honey from Serbia [10], (acacia honey during 2016 (0.16 mS/cm) as well as reported results for honey from Bosnia and Herzegovina, 2016-2017 ( $0.13 \pm 0.15$  mS/cm) [11]. Our results agreed with those reported for acacia honeys from Romania (mean value 0.150 mS/cm) [13].

The insoluble matter is important to detect honey impurities, which includes wax, pollen, honeycomb debris, bees and filth particles. The water-insoluble contents in all honeys ranged from 0.00% to 0.06% with a mean value of 0.01%, and all these values were lower than the permitted maximum limit (0.1%). Similar results of water-insoluble contents were reported in the study of acacia honey from Serbian [10] and from Bosnia and Herzegovina markets [11].

Essentially, honey contains a concentrated water solution of two main sugars, fructose and glucose, with small amounts of various complex sugars. Sugars contribute nearly 95% of honey's dry weight [18], of which 75% is composed of monosaccharides (fructose and glucose), as well as minute amounts of disaccharides (sucrose) and 10-15% of other types of sugars (oligosaccharides and tetrasaccharides). Fructose is always the most important sugar in honey, quantitatively, followed by glucose. Sugars in honey are used for energy supply, as well as contributing to the observed physical

characteristics of honey such as viscosity, hygroscopicity and granulation [19]. Sugar content depends mostly on botanical and to a lesser extent on climate conditions, geographical origin, and on seasonal, processing, and storage conditions [20, 21].

According to [2], the mineral content and the sugar profile have been suggested as criteria for the characterization of monofloral honeys. The sum of glucose plus fructose is a discriminatory variable used to distinguish between blossom and honeydew honeys [1, 5]. The average total value of glucose plus fructose was 69.59% (minimum amount of reducing sugars is 60%). Altogether, 96.94% of tested honeys were in accordance with national regulation [5]. The results were slightly lower than those obtained in studies [11, 22], where the average total value of glucose plus fructose was  $75.47 \pm 0.29$  and  $72.26 \pm 6.53\%$ , respectively. Our previously reported results for acacia honey during 2014, 2015 and 2016 showed the total content of glucose plus fructose ranged from 24.54-71.56%, 23.97-79.13% and 33.42-76.23%, respectively [10].

Besides the reducing sugars analysis, the amount of sucrose is a very important parameter in evaluating the honey maturity. This parameter is analyzed with the purpose of identifying improper manipulation of honey. Inadequate maturation or artificially feeding bees with sucrose syrups can cause high levels of sucrose [22], as does early harvest, which indicates the sucrose was not completely transformed into glucose and fructose [23]. Sucrose was present in all the honeys analyzed, ranging between 0.97 and 42.12%. Only four of the acacia honeys contained higher sucrose content than is recommended (10%) [5]. The mean sucrose content (6.37%) was higher than was reported for other acacia honeys, 2.3% and 2.55%, respectively [11, 12]. According to [24], the reason for the variable levels of sucrose could be due to the transglucosylation reaction initiated by transference of the  $\alpha$ -D-glucopyranosyl unit from sucrose to an acceptor molecule.

The time required for honey to crystallize depends mostly on the ratio of fructose to glucose (F/G), [25]. The F/G ratio is a typical feature of honey types [26]. Honeys that do not crystallize for a long time have F/G ratios greater than 1.33, and if the ratio is less than 1.11, the honey crystallizes quickly [28]. The rate at which glucose crystallization occurs in honey also depends on the glucose/water ratio (G/W), and according to reported data [28, 20], slow crystallization of honey occurred when G/W was less than 1.7, but when it is greater than 2.0, this phenomenon is fast and complete. Glucose is less water soluble than fructose, and therefore, this makes it an important parameter to predict the crystallization tendency of honey [29].

Furthermore, honey crystallization depends on other factors such as the presence of other sugars (e.g. sucrose, maltose), insoluble substances (e.g. dextrin, colloids, pollen), and storage temperature that can influence the crystallization process [30, 31]. The F/G ratio of the examined honeys ranged from 0.44 to 1.91 (Table 1). The mean F/G ratio (1.52) obtained in this study was similar to those reported in other studies [22, 12, 32, 13] ( $1.29 \pm 0.00$ ; 1.40; 1.69-1.82 and 1.50, respectively).

Considering F/G ratio, beside its importance for predicting crystallization, this ratio is also important in testing honey quality as well as authenticity. F/G ratios higher than 1.4 were found to be essential for differentiation between authentic acacia honey and other declared, but unauthentic acacia honeys. Regarding the correlation between glycemic index (GI) and F/G ratio [14], the significance of being able to unequivocally confirm the origin of acacia honey is of great importance, especially for individuals with impaired glucose tolerance or insulin resistance. Sucrose could be partially or totally replaced with honey, particularly low GI honeys, such as acacia, for improving postprandial metabolic response [33].

HMF and diastase activity are routinely used to evaluate honey freshness, providing information about inadequate processing and/or inappropriate storage conditions [34]. Therefore, high quality honey should present high diastase and low HMF contents. The current law stipulates a minimum diastase activity of 8.00 Göthe units. Diastase is a quality factor for determining honey freshness and is influenced by botanical origin, the climate of the region, storage and heating [35]. The diastase activity in the examined honeys ranged between 0.00 and 50.16 Göthe units (mean value 15.79 Göthe units). Lower mean values for diastase activity in acacia honeys were reported in Bosnia and Herzegovina ( $10.19 \pm 8.33$ ) [11] and for those in Serbia in 2014, 2015 and 2016 (13.05; 8.86 and 11.60,

respectively) [10]. HMF is widely known as the most consistent indicator of honey freshness. It is absent in fresh honeys and tends to increase during processing and/or aging [36]. For instance, honeys stored for more than 12-24 months contained 128-1131 mg/kg of HMF and according to study [37], honey should be consumed within one year of storage. Also, high HMF in honeys can be an indication of adulteration by adding invert syrup [38, 39]. Results (mean value 13.18mg/kg, Table 1) showed that the content of HMF in 93.81% of analyzed honeys were below 40 mg/kg and so complied with national and European regulations [5, 1], as well as with some European bee federations that consider a “quality honey” has HMF content lower than 15 mg/kg [40]. However, HMF content in 13 honeys exceeded the legal limit. The mean HMF value was higher than reported data ( $6.79 \pm 1.56$  mg/kg) for China black locust honey [22] and lower than results for acacia honeys from Bosnia and Herzegovina (31.36 mg/kg) [11] and Serbia in 2014, 2015 and 2016 (3.07-140.06 mg/kg; 2.11-387.43 mg/kg and 0.57-211.35 mg/kg, respectively [10]).

#### 4. Conclusion

Government action during 2014-2015 and enhanced quality control probably influenced the better honey quality measured during recent years (2016, 2017 and 2018). Serbia is a honey exporting country, especially of rare and valuable honeys like acacia, sunflower and linden. Adulteration and degradation of the quality of Serbian honey has a significant impact on public health and the economy of the country.

In general, the present results on quality of acacia honeys during 2017-2018, from the entire Serbian market, showed a significant drop of non-compliant honeys (<7% were non-compliant) compared to 2016 and particularly 2014-2015, when the percentage of non-compliant honeys was approximately 20% and 80%, respectively [10].

None of the tested acacia honeys exceeded limits of national or EU regulations [1, 5] for moisture, free acids, insoluble matter, or electrical conductivity. However, similarly to our previous examinations [10], the most relevant parameters for non-compliant honey detection were determinations of HMF, sugar contents and diastase activity. Among these parameters, this study shows the F/G ratio is also an important quality factor and should be defined and taken into consideration for legal regulations on honey quality. Additionally, it could be a cornerstone for rapid confirmation of the authenticity of a honey's floral origin, especially for acacia honey.

Therefore, further research on acacia and other kinds of honey, including on their physicochemical properties and composition is required to guarantee the quality, safety and authenticity of this product and verify its functional and health properties. This work should contribute to current knowledge of acacia honey, and consequently, will support future research to correlate the examined parameters with botanical origin, climate, bee forage, the effects of environment, and production technology on quantity and quality of honey.

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# Bacterial populations and volatile organic compounds associated with meat spoilage

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**Abstract.** The aim of the study was to detect volatile organic compounds acting as markers of spoilage for raw chilled beef stored under vacuum at 4 °C for 15 days. We also determined the relationship of the volatile compounds with microbial and organoleptic properties associated with shelf life. Volatile organic compounds were analysed by multisensory analysis using an electronic nose sensor. Increasing aldehydes, ketones, and alcohols were measured in beef during the storage period. An increase in volatile chemical compounds during storage was correlated with an increased level of *Lactobacillus*, the predominant group of microorganisms on the beef at the end of the study. Maximum concentrations of volatile chemical compounds were determined at the end of the shelf life of the stored beef. Lactic acid bacteria were the main microorganisms that caused spoilage and are suitable for predicting the shelf life of raw chilled vacuum packaged beef using the electronic nose device at the threshold of 5.0-6.0 log CFU/g.

## 1. Introduction

The initial microbial contamination of meat influences the shelf life. The initial microbiota of meat is composed mainly of *Acinetobacter*, *Pseudomonas*, *Brochothrix*, *Flavobacterium*, *Psychrobacter*, *Moraxella*, *Staphylococcus*, *Micrococcus*, lactic acid bacteria (LAB) and *Enterobacteriaceae* [1]. The composition of meat, its favourable pH (5.5-6.5) and its high moisture content ensure this food provides good conditions for microbial growth [2]. There are also external factors such as storage temperature and packaging type that influence the growth of microorganisms on meat during its storage [3].

The combination of chill storage with modified atmosphere packaging or vacuum packaging promotes the growth of certain microorganisms: *Pseudomonas spp.*, *Enterobacteria*, *Brochothrix thermosphacta* and LAB [4]. *Pseudomonas spp.* prevailed over other spoilage microorganisms during aerobic storage at the fridge temperature [5].

The dominant factor in ensuring microbial meat quality and safety is the presence and/or growth of the specific microbial community according to the meat type, meat hygiene and storage conditions [2]. Microbial meat spoilage is caused by the growth of microorganisms that produce metabolites such as aldehydes, ketones, esters, alcohols, organic acids, amines and sulphur compounds.

Currently, numerous studies are aimed at establishing the relationship between organoleptic properties, microbiota and bacterial metabolites (chemical markers) in food products, which can change during storage depending on the type of packaging [6]. Identification of aromatic compounds in meat products that have potential to be used as chemical markers to establish sensory changes during storage was the main aim of these studies [7, 8]. One approach to assessing the shelf life of foods is to evaluate



chemical spoilage indices (CSI). Volatile organic compounds (VOCs), which are metabolites produced by microorganisms, can be used as CSI. The increase of these markers indicates the presence of spoilage microorganisms.

One of the methods used to monitor and measure the gas environment surrounding meat or the gas phase released from the meat on heating is the electronic sensor commonly called the electronic nose [9]. The electronic nose produces a visual imprint of odour resulting from the complex of all substances that form the odour (similar to the human nose).

The main problem in predicting the shelf life of raw meat and meat products is the lack of a clear relationship between the organoleptic and chemical changes that occur during storage. A new model for predicting meat spoilage is needed. A predominant factor for designing the new model is understanding the dynamic changes associated with spoilage caused by microbial communities and their metabolites, and their impacts on sensory qualities of the meat.

## 2. Materials and Methods

### 2.1 Sample preparation

Vacuum packaged, chilled, small-sized beef was examined. The beef was stored chilled with high precision temperature control at  $4^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$  and was examined immediately after production and on days 5, 10 and 15 of storage.

### 2.2 Microbiological analysis

Amounts (10 g) of beef were homogenised in 90 ml of 0.9% saline solution (NaCl) (Vekton, Russia) using a MIX2 homogeniser (AES, France). Then a series of tenfold dilutions were prepared from each initial suspension. Then suitable aliquots were spread on the following growth media: Plate Count Agar (PCA, Merck) incubated at  $30^{\circ}\text{C}$  for 72 h for total microbial count; Crystal-violet neutral-red bile glucose agar (VRBD, Merck) for *Enterobacteriaceae*, incubated at  $37^{\circ}\text{C}$  for 24 h; MRS agar (ФБВН ГИИ ПМБ, Russia) for LAB, incubated at  $30^{\circ}\text{C}$  for 72 h; Dichloran Rose-Bengal Chloramphenicol Agar (DRBC, Oxoid) for yeasts incubated at  $25^{\circ}\text{C}$  for 5 days. Bacterial colony counts were expressed as the means of  $\log_{10}$  CFU/g for all replicates. Identification of microorganisms was performed using the MALDI Biotyper complex on the basis of a desktop time-of-flight mass spectrometer with matrix laser desorption/ionisation (MALDI-TOF) Microflex (Bruker Daltonik, GmbH).

### 2.3 pH measurement

The pH of beef samples (10 g each) was measured using a digital pH meter FiveEasyFE20 (Mettler Toledo, Switzerland) after calibration with commercial buffer solutions at pH 7 and 4. Each sample was homogenised with 100 mL distilled water, and the pH was recorded after signal stabilisation.

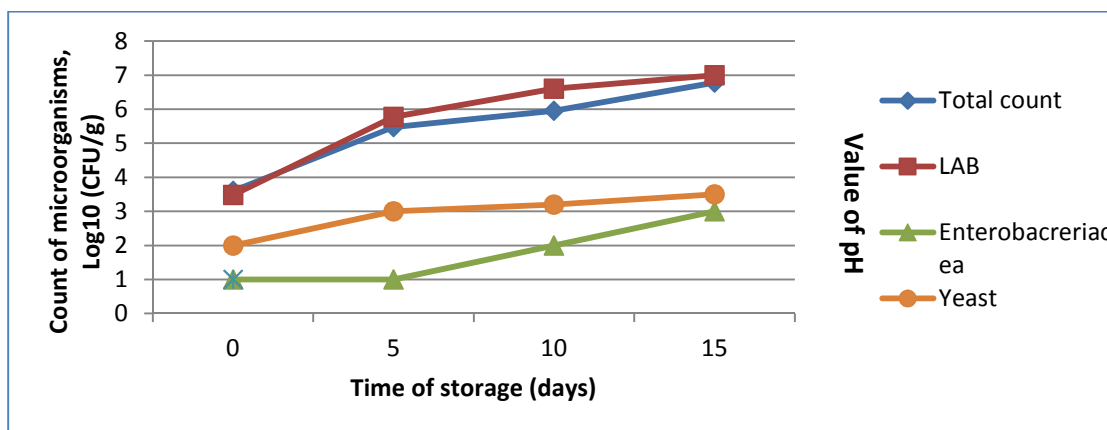
### 2.4 Multisensory analysis of volatile organic compounds

From each piece of beef to be analysed, three samples were taken, one each from the superficial, mid and deep layers. To obtain an average level of VOCs in the beef, each sample under study was crushed, and the required amount was placed in a special glass container (vial). The vial was tightly closed and thermostatically maintained at  $5^{\circ}\text{C}$  for 5 minutes. At the end of the thermostating time, a needle was inserted into the vial to automatically select the gas to be analysed, which was fed to the VOCmeter. The processing of sensor readings (MOS) was performed using the Argus program 2.5.

## 3. Results and discussion

Initially, specific spoilage microorganisms were present at low levels, but they grew faster than other microorganisms and were present at high levels at the end of beef storage. For example, *Lactobacillus* at the initial stage (day 0) numbered less than 1 log CFU/g, but by day 15, had increased to 7.4 log CFU/g.

The maximum microbial levels detected reflected the competition between some of the microbial populations (Figure 1). The total microbial count ranged from 3.6 to 6.7 log CFU/g. The number of other microorganisms (yeasts and *Enterobacteriaceae*) did not exceed 3 log CFU/g throughout the study.

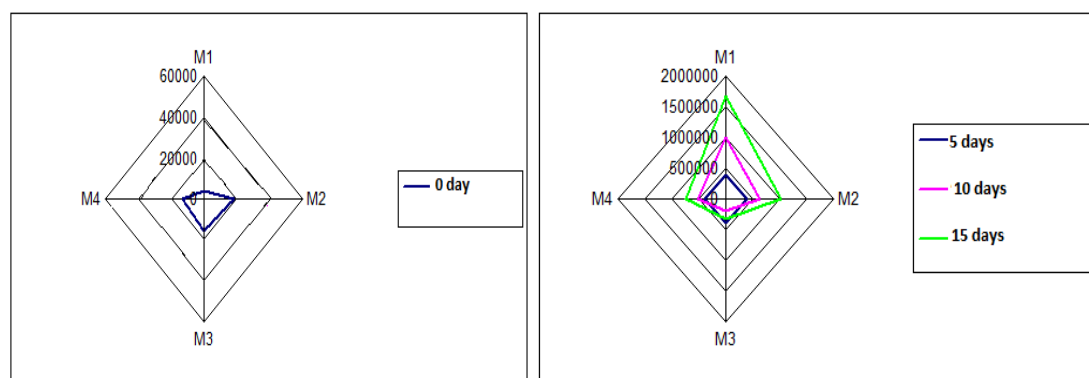


**Figure1.** Microbial populations during anaerobic storage at 4 °C of vacuum packaged beef.

The predominance of *Lactobacillus sakei* among the *Lactobacillus* spp. was established, and *Hafnia alvei* from the *Enterobacteriaceae* family was also identified.

On day 15 of storage, an intensive change in organoleptic characteristics of the beef was determined. This was the appearance of an acidic odour and taste, mucus formation and discoloration. In addition, the content of lactic acid was double that of the control beef examined on day 0, which correlated with the proliferation of *Lactobacillus* during storage. The accumulation of acetic acid was not as intense, but was also increased compared with the level measured at the beginning of the study. The pH of the beef decreased during storage in comparison with the background pH (data not shown).

Organoleptic changes were observed when the count of *Lactobacillus* was 7-8 log CFU/g, which correlated with the results of multisensory analysis of the odour of the beef (Figure 2).



**Figure 2.** Accumulation of volatile organic compounds in vacuum packaged beef stored at 4 °C for 15 days.

In multisensory analysis, a gradual increase in the area of visual imprints (sensors M1-M4, sensitive to aldehydes, ketones and alcohols) was measured, corresponding to the length of storage time. This increase in the amount of VOCs in the gas phase released from the beef was likely associated with the proliferation of the various microorganisms.

Despite this, negative organoleptic changes were observed only at the end of the study (day 15), when multisensory analysis registered high concentrations of VOCs, and the levels of two key microbial indicators were high, i.e. total microbial count (5.9 log CFU/g) and *Lactobacillus* (6.6 log CFU/g).

#### 4. Conclusion

The correlation between increases in chemical markers (VOCs) characterising vacuum packaged beef spoilage and increases in the total counts of spoilage microorganisms (including the prevailing microbial spoilage groups for our anaerobic chilled storage conditions) was established. *Lactobacillus* is proposed as the main group of microorganisms causing spoilage, suitable for predicting shelf life at a threshold level of 5-6 log CFU/g when evaluating the gas phase composition of beef packaged under vacuum and chill stored. Multisensory studies have shown that the sensors in the electronic nose device can detect changes in the odour of raw beef during storage, reflecting the qualitative composition and the quantitative content of substances, depending on the number of microorganisms.

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